University of Alberta

Scoliosis in Pinealectomized Chickens: Understanding the Mechanism and the Relevance to Adolescent Idiopathic Scoliosis

By

Murray Beuerlein ©

A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment of the requirements for the degree of
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The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research for acceptance, a thesis entitled "**Scoliosis in Pinealectomized Chickens: Understanding the Mechanism and Its Relevance to Adolescent Idiopathic Scoliosis**" submitted by Murray Beuerlein in partial fulfillment of the requirements for the degree of Master of Science in Experimental Surgery.

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Abstract

Despite extensive research in many areas there is no known cause for adolescent idiopathic scoliosis (AIS). In addition, there is currently no widely accepted animal model for the study of AIS, which has hampered research. Recently, however, scoliosis has been consistently produced in pinealectomized chickens with several characteristics similar to AIS. The cause of the scoliosis in pinealectomized chickens remains unknown. It has been proposed to result from the lack of melatonin due to the removal of the pineal gland or from an artifact of the surgical procedure other than the removal of the pineal gland.

The results of this thesis indicate that scoliosis develops from the removal of the pineal gland rather than another aspect of the surgical procedure. In addition, the results indicate that serum melatonin levels by themselves are a poor indicator of scoliosis development in pinealectomized chickens. Furthermore, two additional similarities have been identified between the two phenomena. First, as in AIS there is a significant effect of gender on the development of scoliosis in pinealectomized chickens. Second, the development of scoliosis is linked to growth in pinealectomized chickens as in AIS.

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Chapter 1

Introduction

Adolescent idiopathic scoliosis (AIS) is a three-dimensional deformity characterized by lateral curvature of the spine and vertebral rotation (Lonstein, 1994) whose cause remains unknown. Most studies that define scoliosis as a curvature greater than 10° have found the incidence to be 2-3% in the general population (Rinsky and Gamble 1988; Lonstein, 1994). Treatment initially involves simple observation, followed by bracing in an attempt to prevent progression of the curve, and finally surgery in severe cases where progression of the curve has continued despite treatment. Rotation of the vertebrae produces a cosmetic deformity but has more serious implications because if left untreated it can ultimately compromise pulmonary function (Weinstein, 1985).

The spine is a complex of many tissues that are flexible and malleable to external pressure. Consequently, research into the etiology of AIS has focussed on many areas including muscle irregularities (Ford *et al.* 1986), connective tissue abnormalities (Hadley-Miller *et al.* 1994; Jiang *et al.* 1994), proprioception and balance (Barrack *et al.* 1988; Lidstrom *et al.* 1988; Byl and Gray 1993), bone (Cook *et al.* 1987; Saji *et al.* 1995), nervous system disorders (Yamamoto *et al.* 1982; Yamada *et al.* 1984), and irregularities in the growth pattern of patients with AIS (Willner 1976; Skoglund and Miller 1980; Ahl *et al.* 1988; Goldberg *et al.* 1993) to name but a few. Despite this extensive research, the cause of adolescent idiopathic scoliosis remains unknown. Since the cause has remained elusive the focus of scoliosis research has been on treatment methods. While these methods have been successful, they are extensive, invasive and have focussed on treating the symptoms rather than the cause. A better understanding of the mechanism involved in the production of AIS has the potential to revolutionize treatment methods by allowing the focus to shift to the underlying cause.

One factor limiting research into scoliosis has been the lack of an appropriate animal model which is complicated by Man's bipedal stance. Although lateral curves have been created using a variety of techniques in several animal species (Yamada *et al.* 1984; Robin 1990) none has duplicated those seen in AIS (Robin 1990). Recently it has been shown that pinealectomy in young chickens consistently results in the development of scoliosis (Thillard 1959; Dubousset *et al.* 1983; Machida *et al.* 1993, 1994, 1995, 1997; Coillard and Rivard 1996; Kanemura *et al.* 1997; Wang *et al.* 1997, 1998; O'Kelly *et al.* 1999) that has many characteristics similar to those seen in AIS (Machida *et al.* 1994; Kanemura *et al.* 1997; Wang *et al.* 1997).

The mechanism for the production of scoliosis in pinealectomized chickens remains unknown. Results from initial research by Machida *et al.* (1993, 1995) supported the hypothesis that the scoliosis resulted from low levels of serum melatonin, the principal product of the pineal gland because they reported scoliosis in 100% of pinealectomized chickens. Several authors have consistently found the incidence of scoliosis to be about 60% in pinealectomized chickens (Thillard 1959; Coillard and Rivard 1996; Kanemura *et al.* 1997; Wang *et al.* 1997, 1998; O'Kelly *et al.* 1999) even though the levels of serum melatonin in all pinealectomized chickens were found to be significantly reduced, close to zero (Wang *et al.* 1998). These results suggest that pinealectomy in young chickens may result in scoliosis but that serum melatonin levels are not a good predictor of scoliosis development.

The literature is difficult to evaluate because apparently duplicate experiments examining the role of melatonin have produced conflicting results. For example, some experiments involving melatonin therapy and pineal transplantation support the role of melatonin in this phenomenon (Machida *et al.* 1993, 1995) while similar experiments have produced the opposite results (Bagnall *et al.* 1999; Chapter 4). The role of melatonin therefore remains in question and the possibility that other factors may be involved in the phenomenon cannot be dismissed. For example, it has not yet been conclusively shown that the scoliosis does not result from related damage to the cerebral cortex during the surgical procedure rather than the removal of the pineal gland itself. This requires examination because scoliosis in humans is known to result from neuromuscular disorders such as Freidreich's Ataxia or neurofibromatosis (Sussman *et al.* 1996).

The results of the experiments described in this thesis will provide a deeper understanding of the mechanism involved in the development of scoliosis in pinealectomized chickens. The specific objectives of this thesis are to: (1) Determine if scoliosis is a result of the removal of the pineal gland or another aspect of the surgical procedure. (2) Clarify the role of melatonin in this phenomenon. (3) Provide insight into the relevance of the model to AIS. (4) Give direction to future research.

The hypotheses to be tested are:

(1) Scoliosis develops in pineaectomized chickens due to the removal of the pineal gland rather than an artifact of the surgical procedure.

(2) The development of scoliosis in pinealectomized chickens is unrelated to serum melatonin levels.

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Chapter 2

Review of the Literature

2.1 Introduction to Scoliosis

Adolescent idiopathic scoliosis (AIS) is a three-dimensional deformity characterized by lateral curvature of the spine and vertebral rotation (Lonstein 1994) whose cause remains unknown despite extensive research in many areas (Robin 1990). AIS is not uncommon. Most studies that define scoliosis as a curvature greater than 10° have found the incidence to be 2-3% in the general population (Weinstein 1985; Lonstein 1994). Scoliotic curves greater than 20° occur in about 0.5 % of the population and severe curves (>30°) are found in approximately 0. 3% of the population (Lonstein 1994).

Although AIS is often reported to affect predominantly females (Keim 1979) it has been suggested that this is because females tend to develop more severe curves than males (Shohat *et al.* 1988) and are seen more frequently in scoliosis clinics rather than their being an actual gender bias. This is supported by the finding that for 10 – 20° curves the gender ratio is 2:1 in favour of females and when more severe curves are considered (>30°) the ratio increases to 10:1 (Weinstein 1985).

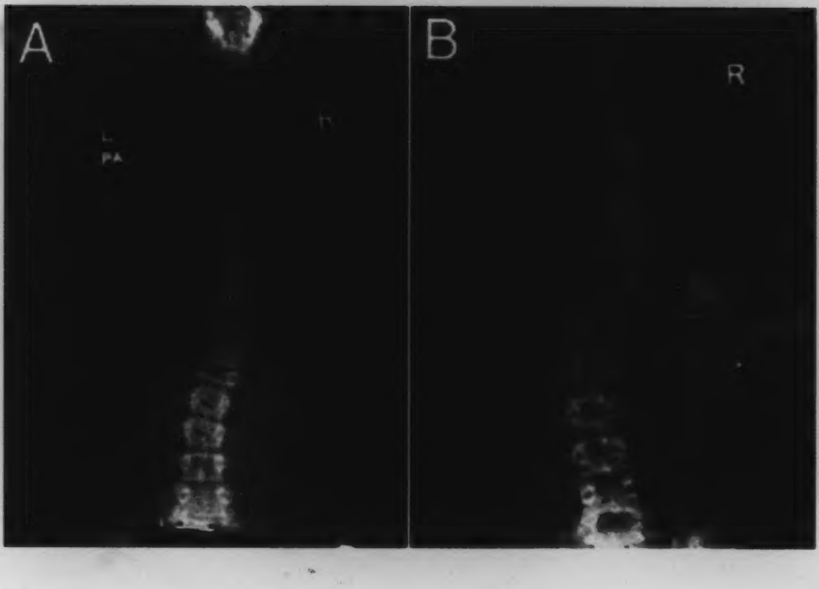
The incidence of scoliosis in adolescents has been found to vary according to parental origin with a higher incidence of scoliosis among adolescents with parental origin from Iraq, Eastern Europe, and Yemen, indicating a possible genetic linkage (Shohat *et al.*, 1988). These data confirm the well established familial relationship that direct relatives of individuals with scoliosis have incidence levels 2-11 times higher than the general population (Wynne-Davies 1968; Reisborough & Wynne-Davies 1973). The familial effect decreased as more distant relatives were surveyed, and the incidence level equaled that of the general population at the third relation (Wynne-Davies 1968).

The vertebral rotation associated with scoliosis is significant because it leads to the development of a rib hump which is the primary cosmetic deformity associated with scoliosis (Raso *et al.* 1997) and if left untreated it may hamper pulmonary function (Weinstein 1985).

Several different curve patterns are common in AIS. The curve can be classified as structural, which is fixed and rigid, or compensatory which is flexible. Structural curves tend to occur in the thoracic spine and compensatory curves are most common in the lumbar spine. The compensatory curve develops to re-establish alignment of the head over the body and will rectify automatically if the structural curve is corrected. In addition, curves can be either single or double (Figure 2-1). More than 90% of single thoracic curves are directed to the right, more than 70% of single lumbar curves are directed towards the left, and more than 90% of all double curves are right thoracic and left lumbar (Rinsky and Gamble 1988).

It is worth mentioning that AIS is diagnosed by exclusion. When all the cases of scoliosis with known causes have been removed, the remaining group is considered idiopathic. It is possible that this idiopathic group may

Figure 2-1. Single (A) and Double (B) curve patterns in adolescent idiopathic scoliosis. L and R signify right and left respectively.


A
L
PA
R
B
R

contain individuals with scoliosis that result from different causes that have not yet been identified.

The progression of the scoliotic deformity is primarily associated with two factors. The first factor is the severity of the curve (Lonstein 1994). Mild curves pose less risk of progression compared to more severe curves (Lonstein 1994). Furthermore double curves have a greater chance of progression than single curves (Lonstein 1994). Second, the child's physical maturity will influence progression. In general the younger, less physically mature a child the more likely the curve will progress (Lonstein and Carlson 1984). This relationship is thought to result because these children have more growth remaining than physically mature patients.

2.2 Treating Idiopathic Scoliosis

The prescribed treatment of idiopathic scoliosis will depend on several factors including (i) degree of curvature, (ii) age or skeletal maturity of the patient, and (iii) flexibility of the curve. Treatment options include simple observation, bracing, and surgery.

Observation is generally prescribed for young patients with mild curves. The physician watches for signs of curve progression and if the curve has progressed more than 10 degrees in the past year a more aggressive approach will be implemented.

Bracing is undertaken when the scoliotic curve is progressing but the patient is too young (i.e. has too much bone growth remaining) or the curve is too mild for surgical intervention. The goal of bracing is not to correct the curve but rather to prevent or limit curve progression while the spine is still growing (Quagliarella *et al.* 1997). The efficacy of bracing scoliosis patients is still under debate. Some studies have found that the success rate of bracing is equal to the proportion of patients who would have not progressed or even regressed without any form of treatment (Miller *et al.* 1984).

Spinal fusion surgery is prescribed for patients who have developed curves of 40 degrees or greater (Lonstein 1994). It is beneficial to delay surgery until most, if not all, spine growth is complete. This prevents the crankshaft phenomenon from developing in which the posterior elements of the spine do not grow because they are fused whereas the anterior region continues to grow. However severe cases often require surgical intervention prior to the cessation of growth because the correction of the curvature is correlated to the flexibility of the spine which decreases with age (Aronsson *et al.* 1996). For a rigid spine, which would only correct minimally, an anterior release procedure can be performed (Matsumoto *et al.* 1997) which increases the flexibility of the spine and facilitates curve correction. Current procedures insert metal rods to fix the spine and allow for the instrumented region of the spine to fuse (Parisini *et al.* 1996). This spinal fusion prevents any further progression of the curve, but also limits mobility in that region. Vertebral rotation is not corrected during the spinal fusion surgery, and therefore the rib hump deformity often remains (Wood *et al.* 1991; Krismer *et al.* 1992).

2.3 Research in to AIS

2.3.1 Factors Complicating Scoliosis Research

There are several factors that complicate and have hampered research into scoliosis. First, the cause of scoliosis seems to be multifactorial with several individual factors being necessary to produce the deformity. Second, AIS is thought by some researchers to be a common end point of numerous conditions (Rinsky and Gamble 1988). Third, there may be two phases in the development of scoliosis. The first phase, corresponding to the initial development of the curve, and the second phase associated with the progression of the curve (Lonstein 1994). Third, researchers are unable to study patients before scoliosis develops and therefore separating cause and effect is difficult. Another factor limiting the advancement of scoliosis research is the lack of an appropriate animal model (Robin 1990) which is made more difficult by Man's bipedal stance. Although scoliosis has been produced in numerous animal models using several techniques the scoliosis has not adequately resembled AIS (Robin 1990). Notably, the models have been unable to duplicate the vertebral rotation seen in AIS.

2.3.2 The Genetics of Idiopathic Scoliosis

Population studies have found a familial pattern in the development of AIS indicative of a genetic link. The mode of inheritance is uncertain but it is thought to be autosomal or sex-linked with incomplete penetrance and expressivity (Lonstein 1994). Some of the current scoliosis research focuses on trying to find a gene that is responsible for the production of scoliosis in humans (Miller *et al.* 1996) but given the multifactorial nature of the deformity, it may be more complicated than identifying a single gene especially if there are several independent causes.

2.3.3 Adolescent Idiopathic Scoliosis as a Structural Disorder

The spine is a biomechanical unit composed of bone, ligaments, intervertebral disks, and muscles. Studies involving all these components in patients with AIS have found significant differences compared to normal controls (Byrd 1987). However it is difficult to differentiate between cause and effect and although the results are interesting they may have to be dismissed on this basis. Consequently, the current consensus is that structural differences between patients with AIS and normal controls are involved in the pathogenesis rather than etiology of the deformity.

2.3.4 Equilibrium and Neurologic Dysfunction.

Several neurological disorders such as Friedreichs Ataxia and neurofibromatosis commonly result in scoliosis (Sussman *et al.* 1996). For that reason it was suspected that patients with AIS may possess mild neurological disorders that are almost non-detectable. This theory is supported by the research of Sahlstrand *et al.* (1978) who found that many patients with scoliosis display diminished vestibular functions, which are exacerbated when vision is occluded. These data implicate a proprioceptive disorder, and the possibility of brain stem dysfunction. In a separate study on the effect of neuromuscular disorders Yamada *et al.* (1984) induced scoliosis in bipedal rats by inflicting them with brainstem lesions. The brainstem is responsible for receiving sensory input from the vestibular apparatus of the ear, and therefore plays a key role in balance. It was proposed that the scoliosis developed due to the loss of vestibular function in these rats. In support of the argument that idiopathic scoliosis arises in individuals with mild neurologic disorders they found that 119 of 150 (79%) of scoliosis patients showed equilibrium dysfunction in at least 1 of 3 tests whereas only 1 of 20 (5%) controls displayed similar equilibrium dysfunction. However, Yamada *et al.* (1984) downplayed the fact that scoliosis produces a body imbalance and that the altered balance functions may be a result of the deformity rather than its cause. Currently research in this area has declined and more attention is being paid to the possibility of hormonal involvement in the etiology of AIS.

2.3.5 Endocrine and Hormonal Differences

2.3.5.1 Growth and Growth Hormone

Adolescent idiopathic scoliosis develops during the rapid growth phase associated with puberty (Willner 1976; Goldberg *et al.* 1993). Therefore aspects of growth have long been postulated to contribute to the etiology of AIS. Numerous studies on the relationship between height and scoliosis have been conducted in the past twenty-five years with Willner being the pioneer (Willner 1974). Most of the early studies compared the height of scoliotic children to the height of age matched controls. The results consistently indicated that girls who developed scoliosis were significantly taller than age-matched controls (Willner 1974; Nordwall & Willner 1975; Dickson & Sevitt 1982; Nicolopuolos *et al.* 1985; Archer & Dixon 1985). Later it was recognized that menarche occurred earlier in scoliotic girls than their non-scoliotic counterparts (Hagglund *et al.* 1992; Goldberg *et al.* 1993). Since the most rapid period of growth in girls occurs prior to menarche (Lonstein & Carlson 1984; Scoles *et al.* 1988) girls with scoliosis reach their peak growth velocity prior to non-scoliotic girls. Goldberg *et al.* (1993) accumulated evidence to support the "Early menarche" hypothesis. Scoliotic girls are taller than age matched controls prior to 13.5 years of age, but are of normal height after that age (Goldberg *et al.* 1993). This change in height pattern can be attributed to non-scoliotic girls "catching up" to the earlier spurt of the scoliotic girls. This was supported by Nordwall and Willner (1975) who found that the growth rate of scoliotic girls was lower than normal girls between the ages of 13 and 15 (Nordwall & Willner 1975). Furthermore, many studies have found the final height of scoliosis patients to be the same as for the normal population (Nordwall & Willner 1975; Ylinkoski *et al.* 1989). It would therefore appear that the increased height of scoliotic girls is due to the early onset of puberty in these girls.

The association between an early growth spurt and scoliosis also explains why females are more susceptible to develop a more severe deformity than males. It is hypothesized that an early growth spurt compromises the stability of the spine in young girls. Males, on the other hand, reach their peak growth velocity two years after females and have more time to develop the stability of their spine. As a result when males reach their peak growth velocity they are able to maintain the stability of their spine.

Growth hormone (GH) is synthesized primarily in the somatotroph cells of the anterior pituitary gland (Greenspan and Baxter 1994). The release profile of GH is episodic (Figure 2-2) (Hartman *et al.* 1991) and responds to the hypothalamic hormones somatostatin (inhibits GH release) and growth hormone release hormone (stimulates GH release). GH directly stimulates the production of insulin-like-growth-factor (IGF-1) which is also known as somatomedin C (Mauras *et al.* 1996). IGF-1 is a potent anabolic hormone that stimulates cartilage and protein synthesis (Burch *et al.* 1986).

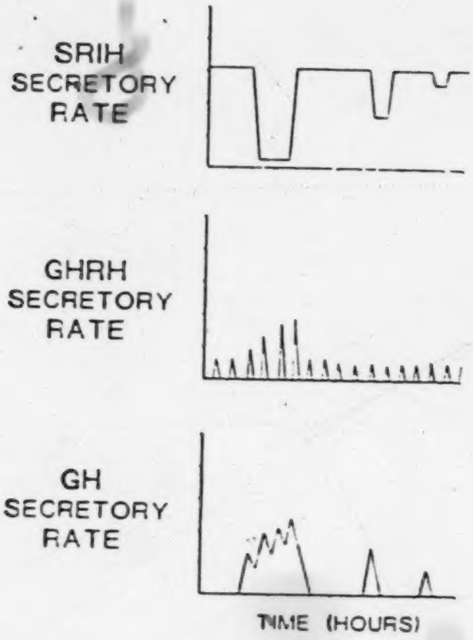


Figure 2-2. The episodic release profile of growth hormone (GH) which is stimulated by growth hormone release hormone (GHRH) and inhibited by somatostatin (SRIH).

Adapted from Hartman et al. 1991

The episodic release pattern of GH complicates the measurement of the hormone. Single samples are of limited value because it is impossible to conclude whether the value obtained corresponds to a peak or a valley on the release curve. Ahl *et al.* (1988) were the first to design an experiment to take the episodic release of GH into account. They obtained 0.5 ml blood samples from patients wit AIS at twenty minute intervals over a twenty-four hour period. By doing so, they were able to show the episodic pattern of GH release and the total integrated GH release over the twenty-four hour period. Puberty is subdivided into five stages, based upon breast and pubic hair development, and they found higher GH secretion in scoliotic girls than control girls, but only during early puberty (Ahl *et al.* 1988). Their findings corroborate the presence of an early growth spurt in girls with scoliosis.

2.3.5.2 The Involvement of Melatonin in AIS

Several studies have looked at the potential role of melatonin in the etiology of AIS. Machida *et al.* (1996) found that AIS patients with progressive curves had significantly lower serum melatonin levels during the night than age matched controls or patients with non progressive curves. In similar studies Bagnall *et al.* (1996) and Hillibrand *et al.* (1996) were unable to detect any differences in the melatonin levels of AIS patients and controls. However, these latter two studies focussed on mature patients with severe scoliosis who had been diagnosed with scoliosis years earlier. A more appropriate time for comparison may be at the onset of the deformity or during curve progression.

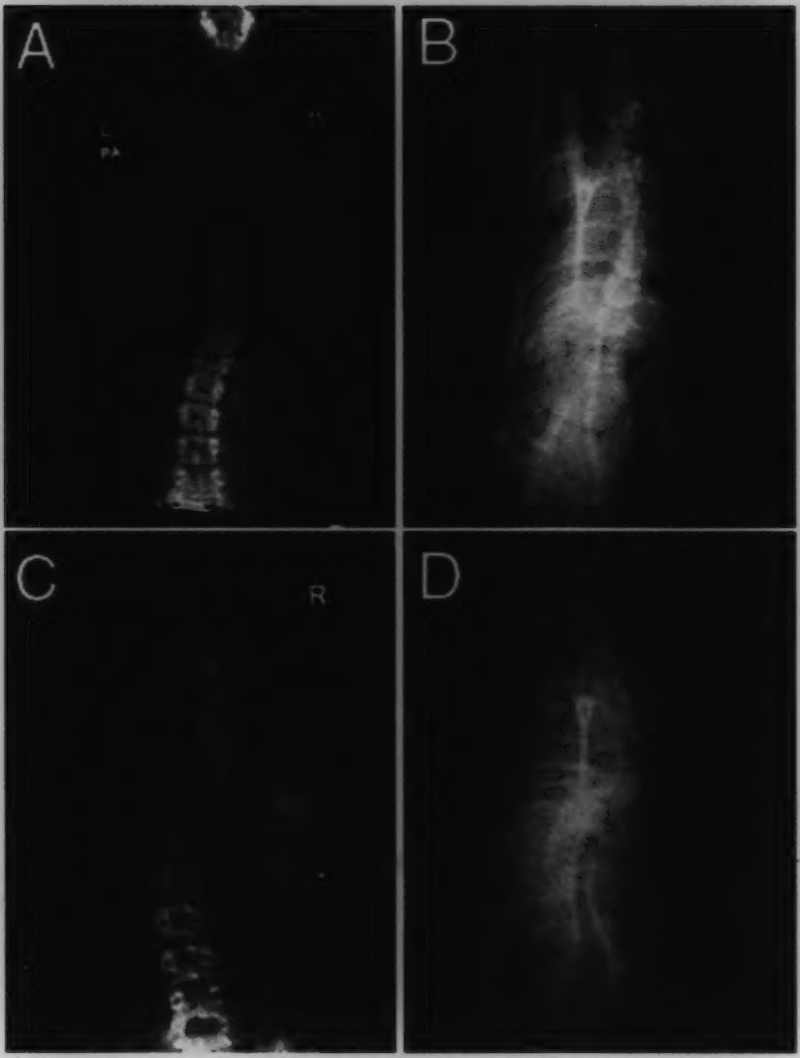
The possibility that low serum melatonin levels are involved in the progression of AIS is exciting because serum melatonin may also be involved in the development of scoliosis in pinealectomized chickens.

2.4 Scoliosis in Pinealectomized Chickens

2.4.1 Introduction

Scoliosis research on pinealectomized chickens began when Thillard (1959) made the apparently serendipitous observation that pinealectomy led to the development of scoliosis. This technique for experimental scoliosis was resurrected by Dubousset (1983) and has received greater attention in this decade (Machida 1993, 1994, 1995, 1997; Coillard and Rivard 1996; Kanemura *et al.* 1997; Wang *et al.* 1997, 1998; O'Kelly *et al.* 1999). Two of the strengths of this model compared to other animal models of scoliosis are that the procedure is not directly aggressive to the spine, and the scoliosis produced closely resembles AIS in humans (Figure 2-3)(Machida *et al.* 1994; Kamenura *et al.* 1997; Wang *et al.* 1997). These similarities include: (1) Scoliosis in pinealectomized chickens is a three-dimensional deformity with both lateral curvature and vertebral rotation (Kanemura *et al.* 1997, Wang *et al.* 1997). In contrast, many of the other animal models have not produced

Figure 2-3. Comparison of scolitic curves between patients with AIS and pinealectomized chickens. (A) Single curve in AIS patient (B) Single curve in a pinealectomized chicken. (C) A double curve in an AIS patient. (D) A double curve in a pinealectomized chicken.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scoliosis with vertebral rotation (Robin 1990). (2) The rib hump deformity is present in pinealectomized chickens with scoliosis (Kanemura *et al.* 1997). (3) The average severity of curve is comparable to AIS (Wang *et al.* 1997). (4) The majority of curves are directed to the right (Kanemura *et al.* 1997; Wang *et al.* 1997). (5) The presence of double and single curves is similar between pinealectomized chickens with scoliosis and patients with AIS (Wang *et al.* 1997). (6) The rate of progression is linked to the magnitude of the initial curve (Kanemura *et al.* 1997). (7) The apex of double curves is found consistently at the same position (T6 in pinealectomized chickens, T9 in AIS) (Wang *et al.* 1997). (8) There is a similar number of vertebrae involved in the scoliosis in pinealectomized chickens and patients with AIS (Wang *et al.* 1997). (9) The development of scoliosis is associated with the growth phase in pinealectomized chickens and AIS (Willner 1976; Goldberg *et al.* 1993; Chapter 5). (10) Serum melatonin levels have been implicated in the development of both (Machida *et al.* 1993. 1995, 1996). These similarities are striking and numerous, and have been the primary reasons why this model has garnered so much attention.

Considering the large phylogenetic gulf between humans and chickens differences might also be expected and are found. However, most of the differences are anatomical rather than differences between the scoliosis. These differences are: (1) There are only seven thoracic vertebrae in chickens compared to 12 in humans (Kanemura *et al.* 1997; Wang *et al.*

1997) and therefore the biomechanics may differ between the two. (2) The lumbar spine of chickens normally has no mobility (Kanemura *et al.* 1997) and therefore the curves in chickens are restricted to the thoracic region (Wang *et al.* 1997). (3) Only the first and sixth thoracic vertebrae show extensive mobility in chickens compared to equal mobility at all thoracic levels in humans (Kanemura *et al.* 1997, Wang *et al.* 1997). (4) The intervertebral disks are substantial in humans, whereas they are small and thin in chickens (Wang *et al.* 1997). As a result of having small intervertebral disks the chicken vertebrae are possibly more susceptible to wedging during scoliosis and wedge prior to curve formation whereas human vertebrae are protected from wedging by the thick intervertebral disk and wedge only after the curve is established (Bagnall *et al.* In Press)

Considering the many important similarities between scoliosis in pinealectomized chickens and AIS, and the seemingly minor anatomical differences the model appears to be appropriate. This is exciting because the development of an appropriate animal model may lead to a better understand of the deformity and new treatment options for AIS.

2.4.2 Mechanism

The mechanism for the production of scoliosis in pinealectomized chickens remains unknown. Initial research by Machida *et al.* (1993, 1995) supported the hypothesis that the scoliosis resulted from low levels of serum melatonin, the principal product of the pineal gland. They were successful in achieving scoliosis in 100% of their pinealectomized chickens, which was reduced to 10% (3/30) when the pineal gland was transplanted into the thoracic musculature (Machida *et al.* 1993) and to 20% (6/30) when melatonin replacement therapy was undertaken (Machida *et al.* 1995).

Unfortunately, these results have yet to be confirmed by other studies. Several authors have found the incidence of scoliosis to be approximately 60% in pinealectomized chickens (Thillard 1959; Coillard and Rivard 1996; Kanemura *et al.* 1997; Wang *et al.* 1997, 1998; O'Kelly *et al.* 1999). However, when the levels of serum melatonin were assayed in these pinealectomized chickens no differences in serum melatonin levels were found between those chickens that developed scoliosis and those that did not (Wang *et al.* 1998). Therefore serum melatonin levels alone are not a good predictor of scoliosis development in pinealectomized chickens. Furthermore, other attempts at melatonin therapy, using a more physiological dose (Bagnall *et al.* 1999) and pineal transplantation (Chapter 4) have proven ineffective in altering the development of scoliosis in pinealectomized chickens. These conflicting results cloud the validity and reliability of earlier experiments and question the role of melatonin in this phenomenon. They also indicate that other factors may be responsible for the development of scoliosis in addition to or separate from serum melatonin levels in pinealectomized chickens.

It has also been suggested that the scoliosis in pinealectomized chickens results from damage to the cerebral cortex during the surgical procedure rather than the removal of the pineal gland itself. This hypothesis has emerged because scoliosis is known to result from neuromuscular disorders such as Freidreich's Ataxia or neurofibromatosis (Sussman *et al.* 1996) and many of the previous results, especially the low incidence of scoliosis could be explained if some aspect of the surgical procedure was responsible for the development of scoliosis. In addition, the inability of melatonin therapy to prevent scoliosis development (Bagnall *et al.* 1999) could be explained if the scoliosis actually resulted from an aspect of the surgery other than the removal of the pineal gland.

Even though the mechanism is unknown, one fact that cannot be disputed is that when the pineal gland is surgically removed many chickens develop scoliosis. It is important to better understand the role of the pineal gland in chickens and humans if the mechanism is to be understood.

2.5 The Pineal Gland and Melatonin

2.5.1 Morphology

The pineal gland belongs to a family of structures known as the circumventricular organs which are located in close proximity to the ventricles of the brain (Greenspan and Baxter 1994). The circumventricular organs are distinct in that they have fenestrated capillaries which permit the diffusion of large molecules between the blood and cerebrospinal fluid (CSF) (Relkin, 1983). This contrasts with elsewhere in the brain where tight junctions produce a blood-brain barrier that is relatively impervious to large molecule diffusion.

2.5.1.1 Mammals

The pineal gland is located deep within the brain of humans, projecting backward from the back of the roof of the third ventricle, where it lies between the posterior edge of the corpus callosum and the superior colliculi (Relkin, 1983). The base of the pineal stalk possesses a recess that is continuous with the cavity of the third ventricle (Snell, 1980). Two types of cells are found in the gland, pinealocytes and glial cells, both of which originate from the neuroectoderm. Pinealocytes are the secretory cells which produce and excrete the products of the pineal gland (Reiter, 1991).

2.5.1.2 Avian Species

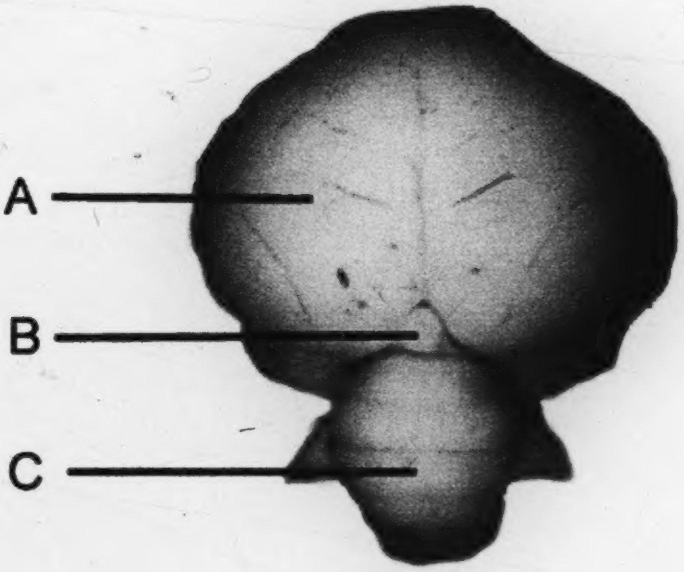
In avian species, the pineal gland is located in the inverted triangular space formed by both hemispheres of the telencephalon and the cerebellum (Figure 2-4). The distal end-vesicle is exposed on the surface of the brain and adheres firmly to the dura mater (Volrath 1981). The proximal portion of the pineal gland, the pineal stalk, is connected to the dorsal wall of the third ventricle.

2.5.2 Innervation of the Pineal Gland

2.5.2.1 Mammals

In mammals, the innervation of the pineal gland is primarily sympathetic via the superior cervical ganglion (SCG) (Laitinen *et al.* 1995). The metabolic activities of the pineal gland can be inhibited by sectioning of the sympathetic

Figure 2-4. An isolated chicken brain showing (A) the cerebral cortex (B) pineal gland and (C) the cerebellum. The pineal gland is located at the point of intersection of the two cerebral hemispheres and the cerebellum.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innervation, or through the use of β-adrenergic antagonists (Nathan *et al.* 1997). Conversely, activation of the sympathetic nervous system causes an activation of pineal metabolism. In mammals, lesions of the suprachiasmatic nucleus (SCN) of the hypothalamus abolish the pineal rhythm indicating that the SCN is the site of the endogenous pacemaker of the circadian rhythm (Casssone *et al.* 1993). Therefore the mammalian pineal gland relies upon proper innervation to receive the circadian rhythm.

2.5.2.2 Avian Species

Like mammals, the avian pineal gland receives post-gangloinic sympathetic innervation from the SCG. However, removal of the SCG does not abolish the circadian rhythm immediately (Ralph 1975; Takahashi *et al.* 1989). In fact, organ cultured avian pineal glands continue to produce and secrete melatonin in a diurnal rhythm for at least three days (Deguchi 1979). These findings indicate that the pineal gland in avian species contains an endogenous pacemaker (Okano and Fukada 1997). Therefore, unlike mammals the avian pineal can continue to produce melatonin in a circadian rhythm without sympathetic innervation but not indefinitely.

2.5.3 Synthesis of Melatonin in the Pineal Gland

The synthesis of melatonin occurs in pinealocytes of the pineal gland along a pathway originating from the essential amino acid tryptophan (Figure 2-5). Other sites of melatonin synthesis include the retina, however only pineal melatonin significantly contributes to serum levels (Wiechmann 1986).

The enzyme serotonin N-acetyl-transferase (SNAT) catalyzes the rate limiting conversion of serotonin to N-acetyl serotonin. The level of this

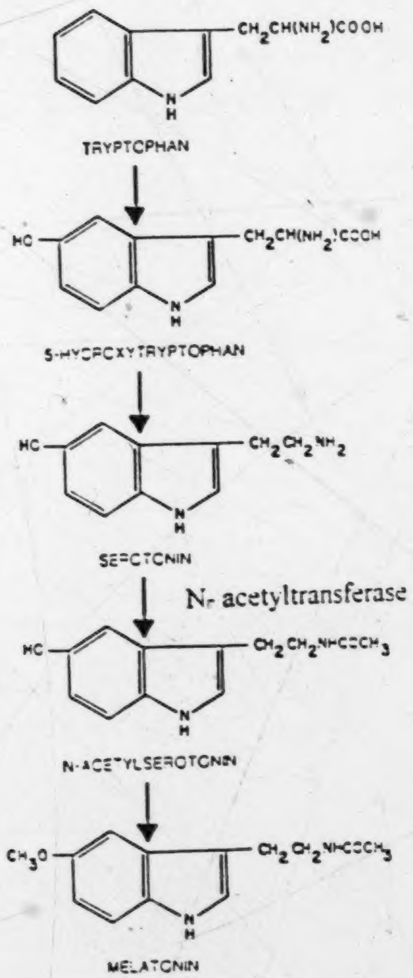
CH2CH(NH2)COOH
TRYPTOPHAN
HO
CH2CH(NH2)COOH
5-HYDROXYTRYPTOPHAN
HO
CH2CH2NH2
SEROTONIN
N- acetyltransferase
HO
CH2CH2NHCOCH3
N-ACETYLSEROTONIN
CH3O
CH2CH2NHCOCH3
MELATONIN

Figure 2-5. The biochemical pathway for the production of melatonin from the essential amino acid tryptophan. N-acetyltransferase catalyzes the rate limiting conversion of serotonin to N-acetyl serotonin.

Adapted from: Hilibrand et al. 1996

enzyme is highest at night and is responsible for establishing the daily rhythm of melatonin. The nocturnal increase in the expression of serotonin N-acetyltransferase varies from 7 - 150 fold in vertebrates (Klein *et al.* 1997). The level of this enzyme decreases rapidly following exposure to light and this is why light exposure rapidly reduces serum melatonin concentrations.

2.5.3.1 Mammals

The level of serotonin N-acetyltransferase responds to sympathetic stimulation as described in Figure 2-6 (Reiter, 1991). The stimulus arises from the suprachiasmatic nucleus (SCN) of the hypothalamus and results in release of norepinephrine (NE) into the extracellular space. On the surface of the pinealocyte the NE can encounter two different receptors. The β-adrenoreceptors are stimulated by NE and account for up to 85 % of the nocturnal increase in serotonin N-acetyl-transferase (Reiter 1991). The binding of NE to the β-adrenoreceptors activates adenylate cyclase which generates cAMP as the second messenger. The cAMP is then responsible for activating a cAMP dependent protein kinase which up-regulates mRNA transcription of the serotonin N-acetyltransferase gene.

The second possible receptor that NE can interact with is the α-adrenoreceptor. This receptor does not directly stimulate serotonin N-acetyltransferase production. However, in conjunction with β-adrenoreceptor activation, a synergistic effect is encountered (Klein *et al.* 1983; Nilsson & Reiter 1989). The α-adrenoreceptors involve the Ca^{++} activated, phospholipid dependent enzyme known as protein kinase C (PKC). It is speculated that PKC amplifies the β-receptor mediated rise in cAMP by phosphorylating G-proteins or adenylate cyclase (Sugden 1989).

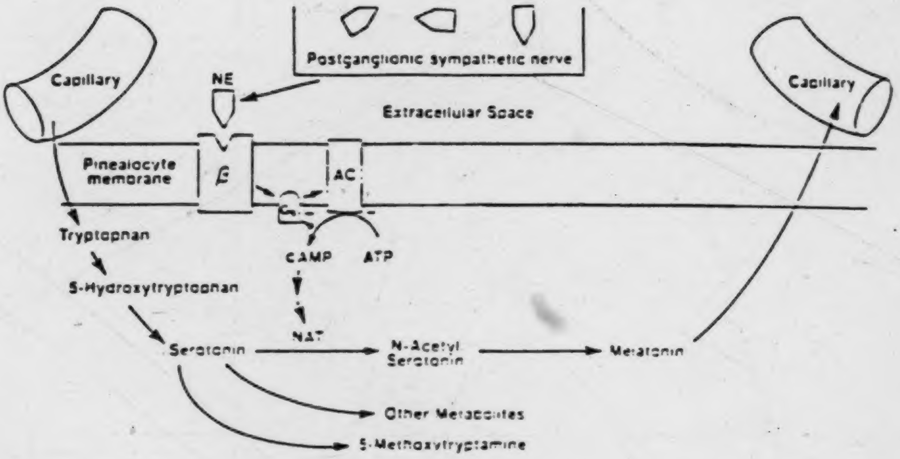
Postganglionic sympathetic nerve
Capillary
NE
Extracellular Space
Capillary
Pinealocyte membrane
ß
AC
G
Tryptophan
CAMP
ATP
5-Hydroxytryptophan
NAT
Serotonin
N-Acetyl Serotonin
Melatonin
Other Metabolites
5-Methoxytryptamine

Figure 2-6. The proposed signal transduction pathway for the control of melatonin production in mammals. Norepinephrine (NE) is released from postganglionic sympathetic nerves and binds to ß-adrenoreceptors (ß) on the pinealocyte membrane. cAMP is produced as a second messenger that up-regulates mRNA transcription of the serotonin N-acetyltransferase (NAT) gene that leads to an increase in melatonin.

Adapted from Reiter 1991.

2.5.3.2 Avian Species

Unlike mammalian species, melatonin production is not regulated by β-adrenergic receptors. As a result the β-adrenergic agonist NE does not illicit an increase in melatonin production in chickens (Blinkley *et al.* 1975). Similarly, β-adrenergic blockers such as propranolol stimulate SNAT production in chickens (Blinkley *et al.* 1978) which is opposite to the effect of β-adrenergic blockers in humans (Nathan *et al.* 1997).

2.5.4 Functions of the Pineal Gland and Melatonin

The functions of the pineal gland are not fully understood, but it is thought to be involved in gonadal development and endocrine rhythms (Cagnacci 1996). These functions of the pineal gland are thought to be mediated via melatonin (Garcia-Patterson *et al.* 1996). Many compounds have been isolated from the pineal gland including biogenic amines, and peptides (Table 2-1). Of these products, melatonin is by far the most abundant (Relkin 1983). Melatonin is highest during childhood, begins to decrease around puberty, and continues to fall until senescence where melatonin levels are minimal (Garcia-Patterson *et al.* 1996).

Evidence for involvement of the pineal gland in gonadal development has mostly been studied in mammals. Low levels of melatonin following pinealectomy have been found to hasten pubertal development in rats (Rivest *et al.* 1985). Similar results have been found in humans where low melatonin concentrations have been seen in precocious puberty (Low *et al.* 1989, Waldhauser *et al.* 1991).

Table 2-1. The Secretions of the Pineal Gland

Biogenic Amine	Peptide
Melatonin	Thyroid release hormone
Serotonin	Somatostatin
Histamine	Vasotocin
Dopamine	Growth Hormone
Octopamine	

Compiled from: Notebom et al. 1993
Greenspan and Baxter 1993

The result of high levels of melatonin are just the opposite. Rats exposed to short day exposure (i.e. activated pineal gland) or exogenous melatonin administration showed delayed sexual maturation (Rivest *et al.* 1985). In humans, high levels of melatonin have been found in women with stress induced (Berga *et al.* 1988) and exercise induced (Laughlin *et al.* 1991) amenorrhea. It is therefore not surprising that melatonin has been used as a female contraceptive in combination with progesterone to prevent ovulation (Voordouw *et al.* 1991). Arendt *et al.* (1989) found high levels of melatonin to be associated with delayed puberty in humans. It would therefore appear that melatonin regulates the onset of development of reproductive system in mammals (Reiter 1983, Ebling & Foster 1989). However, a reverse relationship appears to be true in chickens. Kanemura *et al.* (1997) found that pinealectomized chickens showed underdeveloped cockscombs, and late onset of egg laying. The lack of melatonin in these birds appears to be associated with a delay in reproductive development. In contrast, low melatonin levels in humans were found to be associated with precocious puberty (Low *et al.* 1989, Waldhauser *et al.* 1991).

Other roles of melatonin include an inhibitory role in the development of cancer in humans. This effect appears to be most prominent in sex-hormone dependent tumors such as breast and ovary cancers (Garcia-Patterson *et al.* 1996). Tamarkin *et al.* (1981) found that pinealectomized rats had a higher incidence of induced breast cancer. In addition, they found that the effect was negated by melatonin administration. The inhibitory effect of melatonin on these cancer cells is thought to be linked to its role in gonadal functions.

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Chapter 3

Investigating the Possible Role of an Artifact of the Surgical Procedure in the Development of Scoliosis in Pinealectomized Chickens.

3.1 INTRODUCTION

Pinealectomy in young chickens consistently results in the development of scoliosis (Thillard 1959; Machida *et al.* 1993, 1994, 1995, 1997; Coillard & Rivard 1996; Kanemura *et al.* 1997; Wang *et al.* 1997, 1998; O'Kelly *et al.* 1999) that has many characteristics similar to those seen in patients with adolescent idiopathic scoliosis (AIS) (Machida *et al.* 1994; Kanemura *et al.* 1997; Wang *et al.* 1997). The mechanism behind the phenomenon remains unknown but this animal model is exciting because it has the potential to provide new insights into the development of AIS and to provide the basis for novel treatment methods. Current research has logically focussed on the role of reduced levels of melatonin, the principal product of the pineal gland (Reiter, 1991), following pinealectomy but there has been lingering doubt that the scoliosis might actually be caused by an artifact related to some aspect of the extensive surgery associated with pinealectomy.

Preliminary evidence that a lack of serum melatonin might be the critical factor in the development of scoliosis in pinealectomized chickens was provided by Machida *et al.* (1993, 1995) who showed that both transplantation of the pineal gland into the body wall musculature and melatonin therapy following pinealectomy reduced the incidence and severity of the scoliosis. Such results provide evidence that the phenomenon is not related to an artifact of the surgery, but directly to some element of the pineal gland. Unfortunately, similar and duplicate experiments performed by Bagnall *et al.* (1999) and Beuerlein *et al.* (Chapter 4) have failed to confirm the results and questions their reliability. Furthermore, several studies from different laboratories have shown that only about 60% of pinealectomized chickens develop scoliosis (Thillard 1959; Coillard and Rivard 1996; Kanemura et al. 1997; Wang *et al.* 1997, 1998; O'Kelly *et al.* 1999) even though serum melatonin levels are reduced close to zero in all pinealectomized chickens (Wang *et al.* 1998). Consequently, the role of melatonin in this phenomenon is unclear and the question remains as to whether surgical damage is the underlying cause of this phenomenon. In fact, many of the conflicting results reported in the literature could be explained if some aspect of the surgery was the underlying cause of the phenomenon rather than the simple removal of the pineal gland.

Three experiments to investigate the potential role of surgical artifacts in the development of scoliosis are presented in this chapter. First, the effect of four different pinealectomy techniques on the development of scoliosis was examined to determine if subtle differences in the surgical procedure affected the development of scoliosis. Second, the surgical procedure was performed to varying levels of completion to determine the critical step responsible for scoliosis development. Third, deliberate damage to the cerebral cortex during the surgical procedure was performed to investigate the possible role of such damage in the development of scoliosis.

3.2 METHODS

For three separate experiments, two hundred and sixty eight newly-hatched White Leghorn chickens were obtained from a local hatchery (Lilydale, Edmonton). They were immediately introduced into a 12:12 light / dark cycle (dark 4:00 pm – 4:00 am) and provided food and water *ad libitum*. The chickens were kept in a single pen with constant environmental conditions (26°C, 70% relative humidity). After three days the chickens were randomly assigned to the following experiments and groups.

Experiment 1: Four Different Pinealectomy Techniques

Group 1: Complete pinealectomy in which the pineal stalk was cut distally – near the bulb. n = 21

Group 2: Complete pinealectomy in which the pineal stalk was cut proximally – near the attachment to the brain. n = 20

Group 3: Complete pinealectomy in which the pineal gland was removed using a vacuum hose attached to a pasteur pipette tip. n = 20

Group 4: Complete pinealectomy in which the pineal gland was removed using forceps. n = 19

Group 5: Control chickens that did not undergo any surgical procedure. n = 9

Experiment 2: Identification of the Critical Step of the Pinealectomy Procedure Responsible for the Development of Scoliosis .

Group 1: A control group that did not undergo any surgical procedure. n = 9.

Group 2: Only the skull was cut. By doing so, the superior sagittal sinus and the two transverse sinuses were also cut and the cerebrospinal fluid pressure was reduced as the cranial cavity was opened. n = 20

Group 3: A 'sham' pinealectomy was performed in which the pineal gland was exposed and touched but was not removed. n = 20

Group 4: The pineal stalk was cut but neither the pineal stalk nor bulb were removed. n = 25

Group 5: A complete pinealectomy was performed in which the pineal bulb and stalk were removed. n = 35

Experiment 3: Investigating the Effect of Deliberate Damage to the Cerebral Cortex on the Development of Scoliosis in Pinealectomized Chickens.

Group 1: Pinealectomy – Complete removal of the pineal gland. n=27.

Group 2: Sham pinealectomy – The gland was exposed and touched but not removed. n=17

Group 3: Damage - The pineal gland was exposed and intentional damage to the cerebral cortex adjacent to the pineal gland was carried out using a #18-guage needle attached to a vacuum hose (Figure 3-1). n=16

Group 4: Control - Chickens that did not undergo any surgical procedure. n=10.

Surgical Procedure

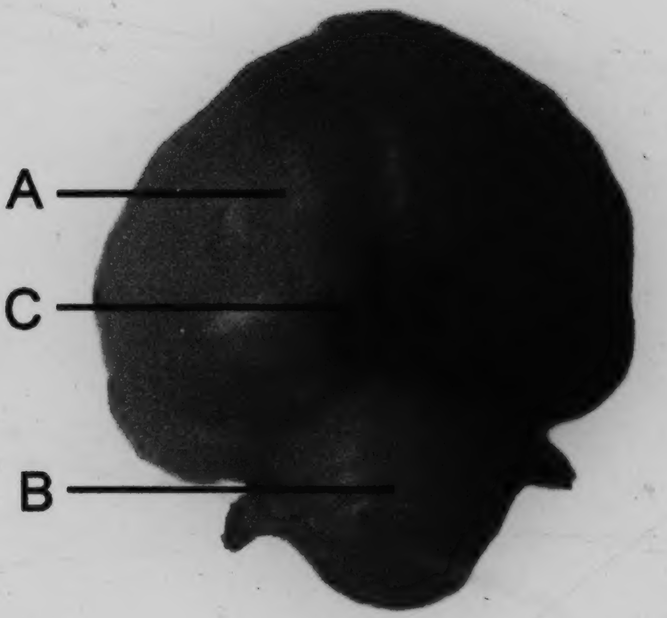
The surgery was performed between three and five days after hatching following the technique described in Appendix A. Modifications to the standard procedure for each group are described above.

After surgery the chickens were placed under a heat lamp and observed until they were fully recovered from the surgery. They were observed for four to five weeks depending upon the experiment during which the incidence of scoliosis and the average serum melatonin levels in each group were determined.

Assessment of Scoliosis

Supine anteroposterior radiographs were taken weekly while the chickens were anaesthetized using halothane. To ensure that the spine remained extended, small weights (115g) were attached to each leg to extend the knees and the chicken's neck was extended by the anaesthetic mask. A previous study has found that the radiographic technique and curve

Figure 3-1. This is a typical example of the extent of intentional damage to the brain immediately after surgery. (A) is the left cerebral hemisphere (B) is the cerebellum, and (C) is the site of intentional damage.


A
C
B

measurements were reproducable to within ± 3 degrees (Bagnall *et al.* Unpublished). The radiographs were examined for the presence of scoliosis with the degree of curvature assessed using the Cobb technique (Cobb 1960). Angles greater than 10° were defined as scoliosis and progressive curves were defined by an increase of 4° or greater on each successive radiograph. Differences in the incidence of scoliosis between groups was tested using Fisher's exact probability test with the level of significance set at $p < 0.05$.

Blood Collection

Blood samples were collected from a wing vein using non-heparinized #18-gauge needles during the middle of the dark cycle, which corresponds to the peak in serum melatonin concentrations (Bagnall *et al.* 1999). The only source of light was subdued red light with an overall intensity of 10 lux. These lighting conditions do not alter the serum melatonin levels in chickens (Reiter 1991; Chapter 7). The time needed to complete blood collection for all 70 chickens each week was less than two hours. The blood was left at room temperature for four hours to allow for coagulation and stored for 16-24 hours at 4° C. The blood samples were cryo-centrifuged for 20 minutes at 3000 rpm at 4° C and the serum stored at -20° C until assayed within two weeks.

Melatonin Assay

The amount of melatonin present in the serum was assessed using a competitive binding ^{3}H-melatonin assay (Appendix B). Each sample was assayed in duplicate to ensure reliability. Differences in the average serum melatonin levels between the four groups was assessed using analysis of variance (ANOVA) followed by Fisher's PLSD ad hoc test with the level of significance set at $p < 0.05$.

Examination of Brains

The chickens in experiment 3 were euthanized 4 weeks after surgery and their brains were exposed using dissecting scissors prior to fixing with neutral buffered formalin. Adhesions between the cortex and dura mater were assessed during microdissection and were classified simply as being present or absent. Differences in the incidence of adhesions between the experimental groups and the relationship between the presence of adhesions and the development of scoliosis were tested using Fisher's exact probability test with the level of significance set at $p < 0.05$.

3.3 RESULTS

The chickens quickly recovered from even the most extensive surgery with no apparent ill-effects as evidenced by their rapid ability to start feeding and drinking. Collection of the blood samples did not appear to affect the chickens and there did not appear to be any effects from the weekly exposure to anaesthetic during radiography. The radiographs were all of good quality and did not present any problems for assessment of scoliosis development.

Experiment 1 : Four Different Pinealectomy Techniques

The pattern of scoliosis development is shown in Table 3-1. There were no significant differences in the incidence of scoliosis between the four experimental groups during the five-week period. After five weeks the incidence of scoliosis was 48% (10/21) in group 1 (Cut High), 45% (9/20) in group 2 (Cut Low), 55% (11/20) in group 3 (Suction), 42% (8/19) in group 4 (Pull with forceps), and 0% (0/9) in group 5 (Control).

The average curve severity did not differ between the four experimental groups after five weeks (Figure 3-2). However, the average curve severity was significantly higher in the four experimental groups compared to the controls since none of the controls developed scoliosis.

The proportion of progressive curves did not differ between the four experimental groups. The incidence of progression was 40% (4/10) in group 1 (Cut High), 44% (4/9) in group 2 (Cut Low), 27% (3/11) in group 3 (Suction), and 25% (2/8) in group 4 (Pull with Forceps).

In summary, none of the curve characteristics measured in this study differed significantly between the four experimental groups that differed in the mode of pineal gland removal.

Table 3-1. The Incidence of Scoliosis in Each Experimental Group During the Five Week Experiment

Group	n	Number (%) of Chickens with Scoliosis				
		Week 1	Week 2	Week 3	Week 4	Week 5
Cut High	21	0%	14%	33%	38%	48%
Cut Low	20	10%	25%	30%	40%	45%
Suction	20	20%	25%	40%	50%	55%
Pull with Forceps	19	11%	32%	37%	37%	42%
Control	9	0%	0%	0%	0%	0%

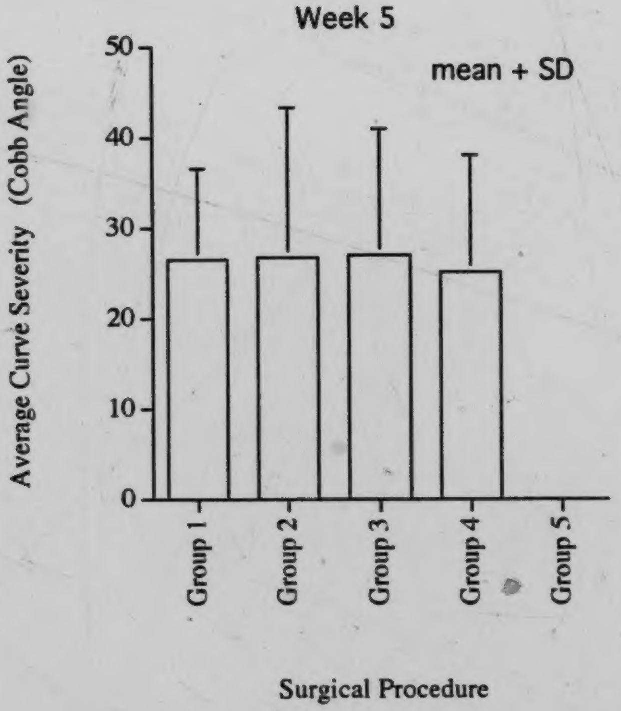


Figure 3-2 The average curve severity five weeks after pinealectomy surgery. There are no significant differences in the average curve severity between groups 1-4, which were all significantly higher than group 5 (Controls). Group 1 (Pineal Stalk cut high), Group 2 (Pineal Stalk cut low), Group 3 (Suction), Group 4 (Pull with forceps), Group 5 (Controls).

Experiment 2: Identification of the Critical Step of the Pinealectomy Procedure.

The results of this experiment show that scoliosis only developed in group 4 (Cut Stalk) and group 5 (Pinealectomy) (Table 3-2). In groups 1, 2 and 3 only 1/49 chickens developed scoliosis (2%) whereas in group 4, 17/25 developed scoliosis (68%) and in group 5, 21/35 developed scoliosis (60%). Apart from the one chicken with scoliosis there was no Cobb angle measurable from the radiographs associated with groups 1, 2, and 3. In groups 4 (Cut Stalk) and 5 (Pinealectomy) the average Cobb angles were not significantly different but were significantly larger than those found in groups 1, 2 and 3 (Figure 3-3). Between weeks 2 and 5 the average Cobb angles for groups 4 and 5 did not differ significantly from each other and progressed equally showing that the development of the scoliosis was similar between these two groups.

During the third week after surgery, the average serum melatonin values in groups 1, 2 and 3 were all normal (Figure 3-4). In contrast, the average values for groups 4 and 5 were both significantly lower than the normal values but not significantly different from each other.

Experiment 3: Investigating the Effect of Deliberate Damage to the Cerebral Cortex on the Development of Scoliosis in Pinealectomized Chickens.

Table 3-2. The Incidence of Scoliosis in the Experimental Groups During the Five Week Experiment

Group	n	Number (%) of Chickens with Scoliosis				
		Week 1	Week 2	Week 3	Week 4	Week 5
Control	9	0%	0%	0%	0%	0%
Cut Skull	20	0%	0%	5%	5%	5%
Sham	20	0%	0%	0%	0%	0%
Cut Stalk	25	0%	24%	64%	68%	68%
Full Pinealectomy	35	0%	26%	57%	57%	60%

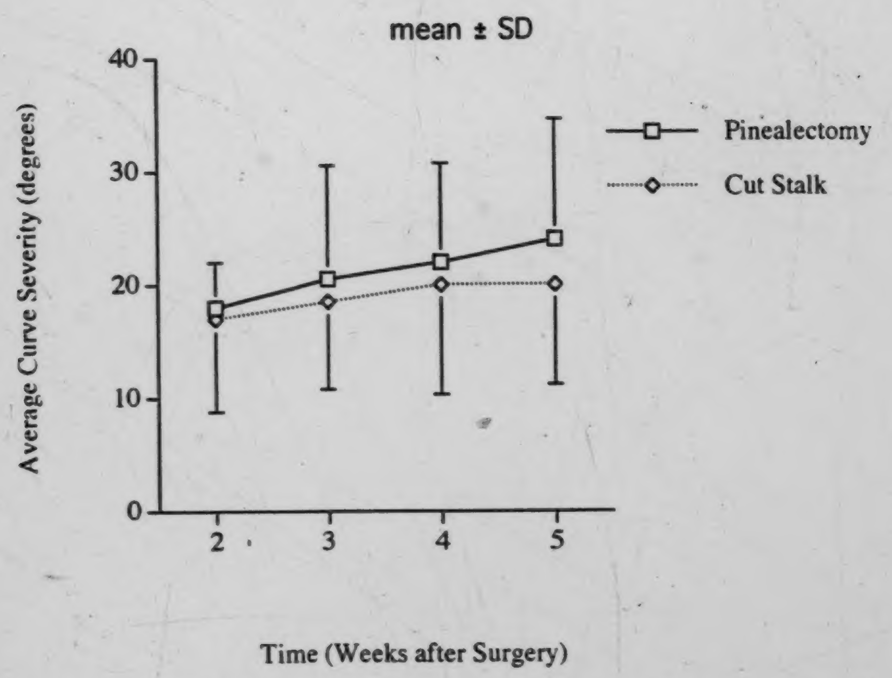


Figure 3-3 The average curve severity during the experiment. No significant differences in curve severity were found between chickens in the Cut Stalk and Pinealectomy group at any period of time.

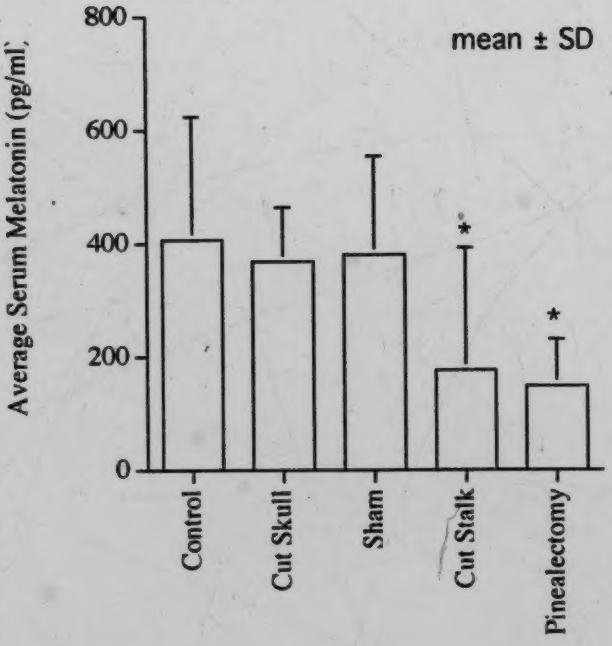


Figure 3- 4 The average serum melatonin levels during the third week after surgery. The average serum melatonin levels of the Cut Stalk and Pinealectomy groups were significantly lower than the Control, Cut Skull or Sham groups. An Asterisk (*) denotes a significant difference from the Control group.

The incidence of scoliosis development is shown in Table 3-3. With the exception of a single chicken, scoliosis was limited only to the group that underwent complete pinealectomy. In the fourth week after surgery the incidence of scoliosis was 56 % (15/27) in the pinealectomy group, 6% (1/17) in the damage group, 0% (0/16) in the sham operation group, and 0% (0/10) in the control group. Interestingly, the average lateral curvature of the chickens in the pinealectomized group that developed scoliosis ($27^\circ \pm 10^\circ$) was similar to the 28° curve seen in the single chicken that developed scoliosis in the intentional damage group.

Overall, eighty-two percent (49/60) of the chickens that underwent any type of surgery developed adhesions between the cerebral cortex and dura mater near the region of the pineal gland. In comparison, 0% (0/10) of the control chickens developed any adhesions. Examples of isolated chicken brains with and without damage due to adhesions are shown in Figures 3-5 and 3-6 respectively. All groups that underwent surgery had a significantly higher incidence of adhesions than the control group (Figure 3-7) and the pinealectomized and damage groups had a significantly higher incidence of adhesions than the sham chickens.

The incidence of adhesions and scoliosis is shown in Table 3-4. Fifty-two percent (13/25) of the pinealectomized chickens with adhesions developed scoliosis compared to 100% (2/2) of the pinealectomized chickens without adhesions that developed scoliosis. When only the chickens that underwent surgery were considered it was found that the incidence of

Table 3-3. The Incidence of Scoliosis Development in the Four Experimental Groups

Group	n	Number (%) of Chickens with Scoliosis			
		Week 1	Week 2	Week 3	Week 4
Pinealectomy	27	0%	41%	56%	56%
Damage	17	0%	6%	6%	6%
Sham	16	0%	0%	0%	0%
Control	10	0%	0%	0%	0%

Figure 3-5. This is a typical example of a chicken brain that had damage to the cerebral cortex in the region of the pineal gland as a result of adhesions between the dura mater and cortex. Most chickens that underwent surgery had adhesions between the cerebral cortex and dura mater resulting in the holes seen here (A).

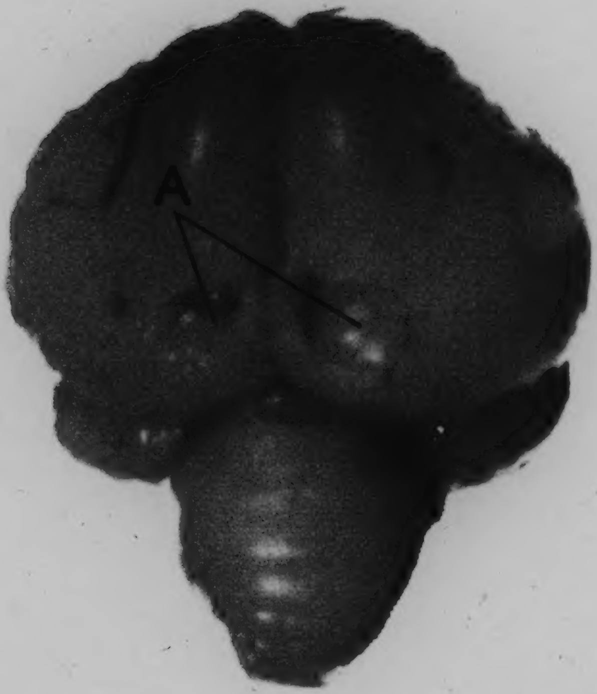
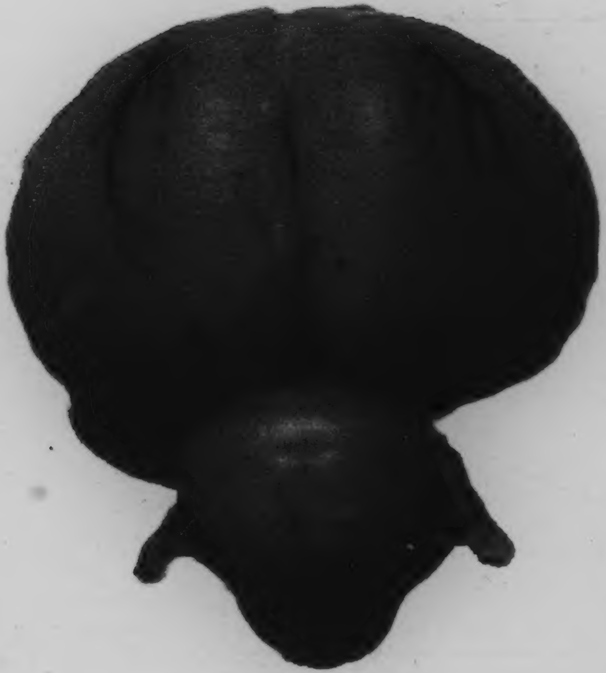

A

Figure 3-6. This is a typical example of a chicken brain that did not have adhesions between the dura mater and cortex. All of the control chickens and some of the chickens that underwent surgery had brains without adhesions to the dura mater.



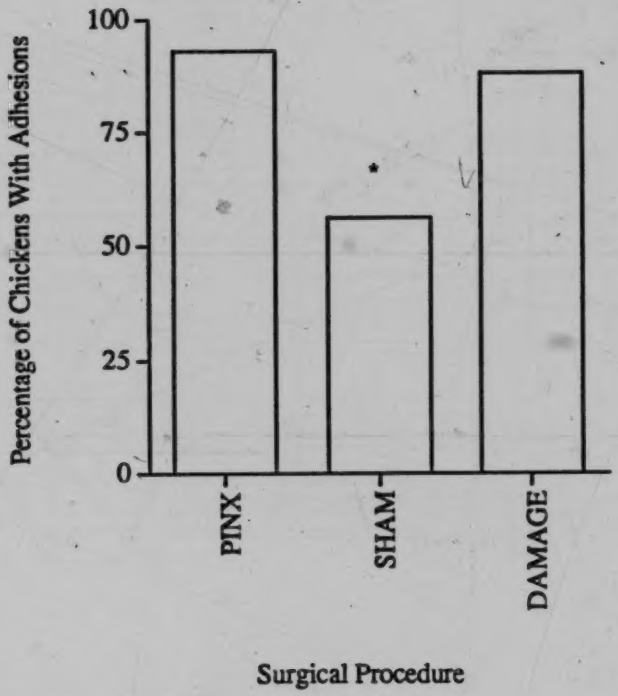


Figure 3-7. The incidence of adhesion between the cerebral cortex and dura mater in the region of the pineal gland. The Pinealectomy (PINX) and Damage groups had a significantly higher incidence of adhesions than the Sham group. The asterisk denotes a statistically significant difference from the PINX and Damage groups.

Table 3-4 The Incidence of Adhesions and Scoliosis in the Chickens after four weeks.

Group	Adhesions	n	Number with Scoliosis
Pinealectomy	Present	25	13
	Absent	2	2
Damage	Present	15	0
	Absent	2	1
Sham	Present	9	0
	Absent	7	0
Control	Present	0	0
	Absent	10	0

scoliosis did not differ between the chickens with adhesions and those without. Twenty-seven percent (13/49) of the chickens with adhesions developed scoliosis, while 25% (3/12) of the chickens without adhesions developed scoliosis.

The serum melatonin levels during the middle of the dark cycle on the third week after surgery are shown in Figure 3-8. The average serum melatonin level of the pinealectomy group was significantly lower than the average serum melatonin levels of either the damage, sham, or control groups which in turn did not differ significantly from each other. The serum melatonin levels during the middle of the dark cycle on the fourth week after surgery are also shown in Figure 3-8. The average serum melatonin level of the pinealectomy group was significantly different from only the control group and again the average serum melatonin levels for the sham, damage, and control groups did not differ significantly from each other.

The serum melatonin level of the one chicken in the damage group that developed scoliosis was 60.4 pg/ml during the third week and 42.0 pg/ml in the 4th week. These levels are within the 95% confidence interval for the damage group for both weeks but outside the 95% confidence interval of the average serum melatonin levels for the pinealectomized chickens which indicates that the pineal gland was functional in that chicken.

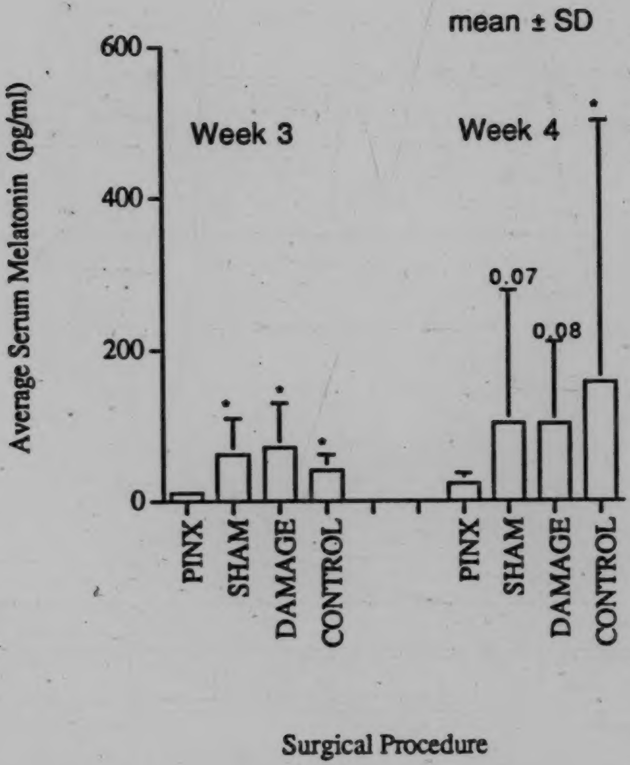


Figure 3-8. Average serum melatonin levels during the third and fourth weeks after surgery. The asterisk (*) denotes a statistically significant difference compared to the pinealectomy group (PINX). The sham, damage, and control groups all have significantly different serum melatonin levels than the pinealectomy group, but do not differ from each other with respect to serum melatonin levels during the third week. In the fourth week the pattern is similar but the difference between the pinealectomy and the sham or damage groups is not significant (p=0.07 and p=0.08 respectively).

3.4 DISCUSSION

The results of experiment 1 indicated that the four pinealectomy techniques that differed in the method of pineal gland removal all produced scoliosis in chickens with similar characteristics. The incidence of scoliosis, average severity of the curve, and number of progressive curves was not significantly different between any of the four groups. It was expected that if scoliosis development was a result of a subtle aspect of the pinealectomy procedure then the four experimental groups would differ in the incidence or severity of scoliosis. However, since no significant differences were detected between the experimental groups it was concluded that the development of scoliosis was not related to a subtle aspect of the procedure.

The results of experiment 2 clearly show that there is a critical step in the pinealectomy procedure before which scoliosis does not develop and after which scoliosis development may occur. When the pineal stalk is cut, the incidence of scoliosis production increases significantly and does not increase further despite more extensive surgery. Furthermore, this step is accompanied by a significant decrease in serum melatonin levels compared to normal values, which again does not change with more extensive surgery. One of the chickens in the group in which only the skull was cut also developed scoliosis but this is not unusual (Wang *et al.*, 1997) especially as it is possible that the pineal stalk was accidentally damaged. This isolated result should not detract from the overwhelming evidence that identifies cutting of the pineal stalk as being the critical step of surgery after which scoliosis can develop. The results also emphasize that removal of the pineal gland is not required for scoliosis to develop, but simply that the pineal stalk needs to be cut.

The results of experiment 3 have shown that the presence of adhesions appear to be a result of the surgical procedure but that these adhesions were not responsible for the production of scoliosis in these chickens because scolsiosis did not develop in the cut skull or sham groups despite the presence of adhesions in those groups. Most importantly, the results also show that scoliosis did not develop when the region of the cerebral cortex near the pineal gland was intentionally damaged during the surgical procedure.

While not providing definitive evidence on their own, the combined results from these three experiments suggest strongly that it is not some artifactual aspect of the surgical procedure that is responsible for the development of scolisois in pinealectomized chickens. Rather, these results indicate that the pineal gland must be disconnected from the brain for scoliosis to develop in these chickens. The focus of attention can again be shifted to the role of pineal products in the phenomenon.

Even though serum melatonin levels were low in all pinealectomized chickens, only about 60% developed scoliosis. These data confirm previous results that showed that low serum melatonin levels do not accurately predict the development of scoliosis in pinealectomized chickens (Wang *et al.* 1998).

If low serum melatonin levels were the critical factor that controlled the development of scoliosis in pinealectomized chickens then presumably all chickens with low serum melatonin levels would be expected to develop scoliosis. It has been proposed that melatonin is not directly involved in the production of scoliosis in pinealectomized chickens but rather indirectly through its relationship with growth hormone (GH) (Machida *et al.* 1995). Considering the established relationship between growth and scoliosis in humans (Willner *et al.* 1976; Hagglund *et al.* 1992; Goldberg *et al.* 1993) and specifically the role of growth hormone in AIS (Ahl *et al.* 1988) the potential role of GH in the development of scoliosis in pinealectomized chickens deserves further attention.

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Chapter 4

The Effects of Pineal Transplantation on the Production of Scoliosis in Pinealectomized Chickens.

4.1 INTRODUCTION

Adolescent idiopathic scoliosis (AIS) is a deformity characterized by lateral curvature of the spine and vertebral rotation (Lonstein 1994) whose cause remains unknown. One of the factors limiting research in this area has been the lack of an appropriate animal model (Robin 1990). However, scoliosis that closely resembles AIS has recently been consistently produced in young chickens following pinealectomy (Machida *et al.* 1993, 1994, 1995, 1997; Wang *et al.* 1997, 1998; O'Kelly *et al.* 1999). This model is exciting because it has the potential to provide further insights into the etiology and pathogenesis of scoliosis and potentially result in novel treatment options.

The mechanism for the production of scoliosis in pinealectomized chickens remains unknown. Initial research by Machida *et al.* (1993, 1995) found scoliosis development in 100 % (30/30) of pinealectomized chickens. They assumed the cause to be reduced levels of serum melatonin, the principal product of the pineal gland (Reiter 1991), although serum melatonin levels were not measured. In contrast, other researchers have consistently produced scoliosis in only approximately 60% of pinealectomized chickens (Thillard 1959 66% (33/50); Coillard and Rivard 1996 80% (20/25); Kanemura *et al.* 1997 68% (17/25); Wang *et al.* 1997 60% (18/30), 1998 52% (17/33); O'Kelly *et al.* 1999 48% (10/21)). Wang *et al.* (1998) found that serum melatonin levels were low in all pinealectomized chickens indicating that serum melatonin levels were not a useful predictor of scoliosis development in pinealectomized chickens and questioning the role of melatonin in this phenomenon.

Machida *et al.* (1995) provided evidence to support the role of melatonin by administering melatonin to pinealectomized chickens. This therapy was successful in reducing the incidence of scoliosis if started immediately after surgery and also reduced the severity of scoliosis if a curve had already developed (Machida *et al.* 1995). Conversely, Bagnall *et al.* (1999) using a more physiologically determined therapy regimen found that melatonin therapy was ineffective in altering the incidence or severity of scoliosis in pinealectomized chickens. This series of conflicting results is curious and the role of melatonin in this phenomenon remains in question.

It has also been suggested that scoliosis in pinealectomized chickens has not been produced by the removal of the pineal gland but by some other aspect of the extensive surgery, perhaps by inadvertently damaging the adjacent cerebral cortex. The melatonin therapy results described by Machida *et al.* (1995) would have eliminated this latter possibility but Machida's results have yet to be confirmed (Bagnall *et al.* 1999). Another experiment, which might eliminate the role of surgery in the phenomenon, would be to transplant the pineal gland elsewhere in the body following pinealectomy and observe the development of scoliosis. Machida *et al.* (1993) autografted the removed pineal gland into the thoracic musculature in pinealectomized chickens. Only 10% (3/30) of these chickens developed scoliosis compared to 100% (30/30) of the pinealectomized chickens that did not receive a grafted pineal gland. These results suggested that the scoliosis developed from a lack of pineal function rather than some aspect of the surgery, although serum melatonin levels were not measured and pineal function was not determined. The literature on pineal transplantation in mammals indicates that transplantation to the anterior chamber of the eye is the only effective means of re-establishing pineal function (Wu *et al.* 1993) and similar results are expected in avian species. Therefore it is difficult to understand how transplantation of the pineal gland to the thoracic musculature could lead to normal serum melatonin levels and the prevention of scoliosis development.

The following experiment was designed to answer some of the questions that remain concerning pineal transplantation into the thoracic musculature in pinealectomized chickens. The two objectives were to: (1) Assess the viability of pineal transplants to the thoracic musculature in pinealectomized chickens by measuring serum melatonin. (2) Determine the incidence of scoliosis in pinealectomized chickens that received a pineal transplant.

4.2 METHODS

One-hundred-and-forty-six, newly-hatched chickens were obtained from a local hatchery (Lilydale, Edmonton). The chickens were kept in a single pen with constant environmental conditions ($26^{\circ}C$ and 70% relative humidity) throughout the experiment. They were immediately introduced into a 12:12 light / dark cycle (dark 9:00 am – 9:00 pm) and provided food and water *ad libitum*. The chickens were randomly assigned to the following groups.

Group 1	n = 43	Pinealectomy
Group 2	n = 43	Cut Stalk
Group 3	n = 35	Transplantation
Group 4	n = 25	Control

The surgical procedure was performed between three and five days after hatching. Group 1 (Pinealectomy) underwent the complete removal of the pineal gland (Appendix A). The pineal gland was exposed by making an incision in the skull and removed with forceps after the pineal stalk had been cut. Group 2 (Cut Stalk) underwent the same procedure except that after the pineal stalk was cut the pineal gland was left *in situ* attached to the dura mater. This group was considered a pseudo-transplant group in which the gland was left *in situ*. Group 3 (Transplantation) underwent a complete pinealectomy procedure after which an incision was made in the anterior chest wall and the pineal gland was placed in a small incision made in the pectoral muscle. Group 4 (Control) did not undergo any surgical procedure.

After surgery the chickens were placed under a heat lamp and observed until they were fully recovered. Afterwards the chickens were returned to their normal environment and were randomly assigned to either the short-term (Part A) or the long-term study (Part B).

Part A: Short Term Study

This phase of the experiment was designed to determine the serum melatonin levels of the chickens one week after surgery. The melatonin assay requires 1 ml of blood to be collected which is approximately 50% of the total blood volume of a 1 week old chicken and removal of such a large amount of blood would be fatal. Therefore chickens had to be sacrificed to determine the serum melatonin levels one week after surgery.

Group 1	n = 8	Pinealectomy
Group 2	n = 5	Cut Stalk
Group 3	n = 10	Transplantation
Group 4	n = 11	Control

Blood Collection

On the seventh day after the surgery, the chickens were euthanized using 2.5 ml euthanyl per kilogram body weight and 1 ml of blood was withdrawn from the heart using non-heparinized #18 gauge needles. The samples were collected during the middle of the dark cycle because that is when serum melatonin levels are at their peak (Bagnall *et al.* 1999). Dim red lights (~ 4 lux) were the only source of light during blood collection. These lighting conditions were chosen because they did not alter the serum melatonin levels in chickens (Reiter, 1991; Chapter 7). The blood was left at room temperature for four hours to allow for coagulation to occur and stored for 16-24 hours at 4° C. The blood samples were cryo-centrifuged for 20 minutes at 3000 rpm at 4° C and the serum stored at –20° C until assayed within two weeks.

Part B: Long Term Study

The remaining one hundred and twelve chickens were observed until five weeks after hatching to determine the incidence of scoliosis following surgery and the serum melatonin levels during weeks 3 – 5 post operatively.

Group 1	n = 36	Pinealectomy
Group 2	n = 37	Cut Stalk
Group 3	n = 25	Transplantation
Group 4	n = 14	Control

Blood Collection

Blood samples were collected in the middle of the dark cycle from a wing vein using a non-heparinized #18 gauge needle. The only sources of light were subdued red lights with an average intensity of 10 lux. The time needed to complete blood collection for all 112 chickens was less than two hours each week. The samples were then treated as described previously.

Melatonin Assay

Serum melatonin levels were assessed using a competitive binding ^{3}H-melatonin assay (Appendix B). Each sample was assayed in duplicate and the average value was used for the analysis. Differences in the average serum melatonin levels among the four groups were assessed using analysis of variance (ANOVA) followed by Fisher's PLSD ad hoc test with the level of significance set at $p < 0.05$.

Radiography

Supine anteroposterior radiographs were taken weekly while the chickens were anaesthetized using halothane. To ensure that the spine was extended, small weights (115 g) were attached to each leg and the neck was held extended by the anaesthetic mask. A previous study has found that the radiographic technique and curve measurements were reproducable to within ± 3 degrees (Bagnall *et al.* Unpublished). The radiographs were examined for the presence of scoliosis with the degree of curvature assessed using the Cobb technique (Cobb, 1960). A curve greater than 10° was defined as scoliosis. In addition, the pattern of curvature was determined to be progressive if curves showed an increase of 4° or greater on each consecutive radiograph. Differences in the incidence, severity and progression of scoliosis among the groups were tested using ANOVA followed by Fisher's PLSD ad hoc test ($p < 0.05$). Differences in the incidence of scoliosis and number of progressive curves were tested using Fisher's exact probability test ($p <0.05$).

4.3 RESULTS

The surgical procedure produced no adverse effects on the chickens. On average, chickens took approximately one hour to recover after which time they appeared to feed and behave normally.

Part A: Short Term Study

The average serum melatonin levels for the chickens that were sacrificed one week following surgery are shown in Figure 4-1. The chickens that underwent pinealectomy, pineal transplant and simple cutting of the pineal stalk all demonstrated the same average serum melatonin levels, all of which were significantly lower than those obtained from the controls.

Part B: Long Term Study

From the radiographs it was determined that none of the control chickens (0/14) developed scoliosis (Table 4-1). During the experiment 55% (54 / 98) of the total number of chickens that underwent any type of surgery developed scoliosis. This consisted of 58 % (21 / 36) of the pinealectomy group, 46 % (17 / 37) of the transplantation group, and 64 % (16 / 25) of the cut stalk group. There were no significant differences in the incidence of scoliosis among each experimental group at any period of time. However, the incidences of scoliosis in all the surgical groups were significantly higher than the control group.

The average severity of scoliosis for the three surgical groups (Cobb angle) is shown in Figure 4-2. The control group is not included because none of the control chickens developed scoliosis. No significant differences in curve severity existed among any of the surgical groups during the five-week period. There were also no significant differences in the proportion of

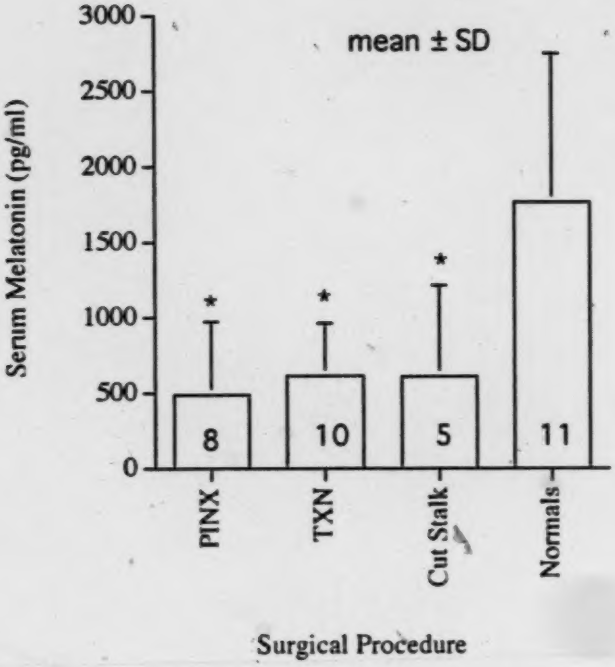


Figure 4-1 . The average peak serum melatonin levels one week following surgery. The asterisks (*) denotes a statistically significant difference from the control group. The number in each column indicates the number of chickens in each group. PINX = pinealectomy group. TXN = pineal transplant group.

Table 4-1. The Incidence of Scoliosis in Each Experimental Group During the Five Week Experiment

Group	n	Number (%) of Chickens that Developed Scoliosis				
		Week 1	Week 2	Week 3	Week 4	Week 5
Pinealecomy	35	0%	36%	49%	58%	58%
Transplantation	38	0%	30%	41%	43%	44%
Cut Stalk	25	0%	40%	56%	60%	64%
Control	14	0%	0%	0%	0%	0%

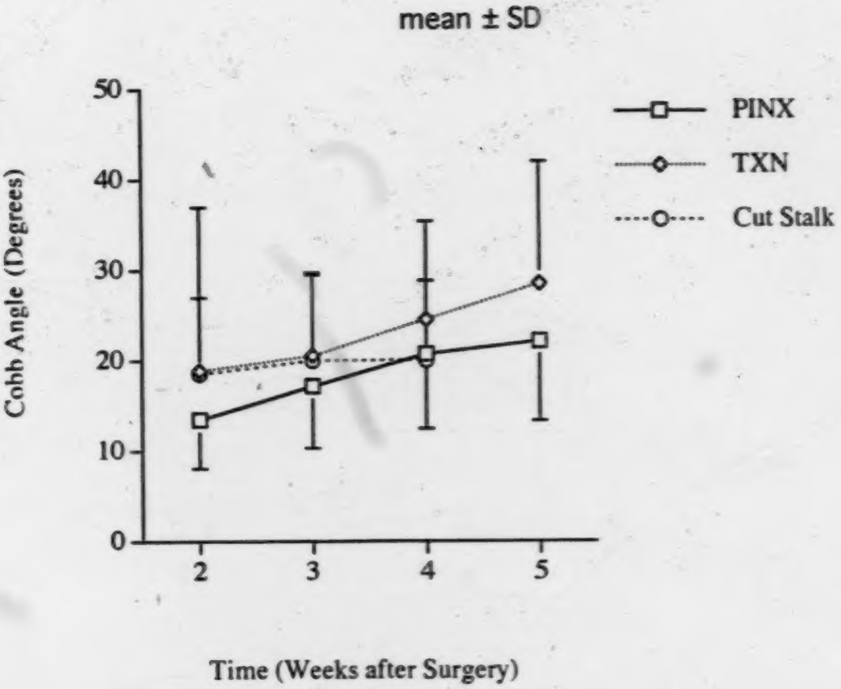


Figure 4-2 . The average Cobb angle for those chickens that developed scoliosis. No significant differences were detected between any of the experimental groups at any period of time. PINX = pinealectomy group. TXN = pineal transplant group.

progressive curves among the three surgical groups. The pinealectomy group had 30% (6/20) of the curves classified as progressive, compared to 35% (6/17) of the transplant group, and 31% (5/16) of the cut stalk group. These results indicate that the pattern of curve development in the three surgical groups was similar throughout the experiment.

For analysis of serum melatonin, each experimental group was subdivided based on whether or not the chickens developed scoliosis. Within each experimental group the average serum melatonin levels for the chickens that developed scoliosis were never significantly different from the serum melatonin levels of the chickens that developed scoliosis (Figure 4-3). Furthermore at all times the experimental groups displayed significantly lower serum melatonin than the controls. The only exception to this occurred in the fifth week when the cut stalk group that developed scoliosis did not differ significantly from the control group (Figure 4-3).

4.4 DISCUSSION

The results of this study showed that both the incidence and pattern of curve development in pinealectomized chickens were not altered by either the transplantation of the pineal gland into the thoracic musculature (Transplant, Group 2) or by *in situ* transplantation of the gland (Cut Stalk, Group 3) when compared to the group of pinealectomized chickens in which the pineal gland was completely removed. In all three experimental groups the incidence of scoliosis was approximately 60%, which agrees with the incidence of scoliosis

Figure 4-3. The average peak serum melatonin levels three, four, and five weeks following surgery. The asterisks (*) denotes a statistically significant difference from the control group.

PINX non = Pinealectomized chickens that did not develop scoliosis. n=12

PINX Scol = Pinealectomized chickens that did develop scoliosis. n=17

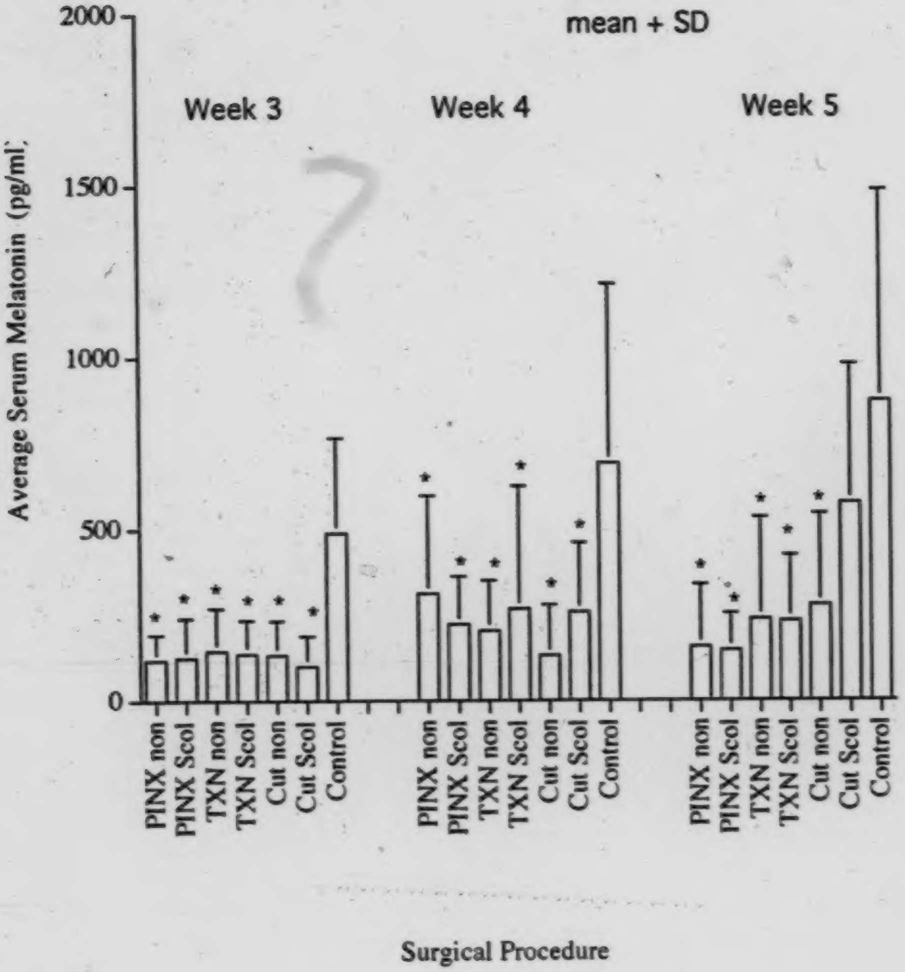
TXN non = Pineal transplantation chickens that did not develop scoliosis. n= 18

TXN scol = Pineal transplantation chickens that did develop scoliosis. n=14

Cut non = Cut stalk chickens that did not develop scoliosis. n=5

Cut Scol = Cut stalk chickens that did develop scoliosis. n=6

Control. n=13.


Average Serum Melatonin (pg/ml)
2000
1500
1000
500
0
mean + SD
Week 3
Week 4
Week 5
PINX non
PINX Scol
TXN non
TXN Scol
Cut non
Cut Scol
Control
Surgical Procedure

found in other studies (Thillard 1959, Coillard & Rivard 1996; Kanemura *et al.* 1997; Wang *et al.* 1997, 1998; O'Kelly *et al.* 1999) but is well below the 100% scoliosis in pinealectomized chickens reported by Machida *et al.* (1993, 1995, 1997).

One week after surgery the serum melatonin levels of the pinealectomy, transplantation, and cut stalk groups were all significantly lower than the serum melatonin levels of the control group, indicating that chickens in those groups lacked a functioning pineal gland. This pattern was repeated for all of the subsequent weeks. Therefore, pineal transplantation into the thoracic musculature or *in situ* appears to be ineffective in restoring normal pineal function as measured by serum melatonin levels.

The serum melatonin levels within each group (Pinealectomy, Transplant, Cut Stalk) did not differ between those chickens that developed scoliosis and those that did not. However, in these groups only 55% (54/98) of the chickens developed scoliosis. These data confirm previous results that showed that low serum melatonin levels do not accurately predict the development of scoliosis in pinealectomized chickens (Wang *et al.* 1998). If low serum melatonin levels were the critical factor that controlled the development of scoliosis in pinealectomized chickens then all chickens with low serum melatonin levels would be expected to develop scoliosis.

Unlike Machida *et al.* (1993) who found that the incidence of scoliosis was reduced to 10% (3/30) in pinealectomized chickens receiving an intra-muscular pineal transplant, the results of the present study indicate that intramuscular and even *in situ* pineal transplantation is ineffective in altering the development of scoliosis in pinealectomized chickens. Similarly, the curve severity was not affected by pineal transplantation in this study. No significant differences in curve severity were found among any of the surgical groups during the five-week period. This contrasts sharply with the results described by Machida *et al.* (1993) who reported a decreased curve severity within the group that received the pineal transplantation. These conflicting results suggest that the pineal transplant by Machida *et al* (1993) was effective in restoring pineal function whereas the pineal transplantation in this study was not. However, there is no evidence to support the idea that intramuscular or *in situ* transplantation is viable (Wu *et al.* 1993). Innervation is an essential component to re-establishing normal serum melatonin levels in both mammalian and avian species. The endogenous circadian oscillator within the pinealocytes of avian species can maintain the cycle without proper innervation but only for a short period of time (Deguchi 1979; Okano and Fukada 1997). Wu *et al.* (1993) found that transplantation to the anterior chamber of the eye was the only effective means of re-establishing proper innervation and restoring normal pineal function. It is not known how the pineal transplantation performed by Machida *et al.* (1993) was able to restore pineal function and prevent scoliosis.

In conclusion, pineal transplantation into the thoracic musculature in pinealectomized chickens appears to be ineffective in restoring normal pineal function as measured by serum melatonin levels. In addition this study confirmed previous results of Wang *et al.* (1997, 1998) in that chickens with normal serum melatonin levels did not develop scoliosis, whereas chickens with low serum melatonin levels were susceptible to develop scoliosis with an incidence of about 60%. The results of this study do not clarify the role of the surgery in the production of the scoliosis. Further research to definitively rule out the surgical procedure as the cause of the scoliosis remains.

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Chapter 5

The Development of Scoliosis Following Pinealectomy in two Species of Chickens with Different Rates of Growth

5.1 INTRODUCTION

Adolescent idiopathic scoliosis (AIS) is a three dimensional deformity characterized by lateral curvature of the spine and vertebral rotation (Lonstein 1994). Despite extensive research the cause remains unknown but several facts have been established. For instance, AIS develops during the rapid growth phase of puberty (Ahl *et al.* 1988; Goldberg *et al.* 1993), and therefore aspects of growth have long been postulated to contribute to the etiology of AIS. Studies on the relationship between height and scoliosis have found that girls who developed scoliosis were significantly taller than age-matched controls (Willner 1974; Nordwall & Willner 1975; Nicolopuolos *et al.* 1985; Archer & Dixon, 1985) but only during puberty because final height between the two groups was the same (Goldberg *et al.* 1993). It was also recognized that menarche occurred earlier in scoliotic girls than their non scoliotic counterparts (Hagglund *et al.* 1992; Goldberg *et al.* 1993) signaling earlier maturity and its accompanying growth spurt. These results have been supported by Ahl *et al.* (1988) who found higher growth hormone (GH) secretion in scoliotic girls than control girls but only during early puberty. The evidence therefore supports the concept that growth is an integral part of scoliosis development and that its role must be included in any animal model relating to AIS.

Lateral curves of the spine have been produced in several species using a variety of techniques but none have duplicated those seen in AIS (Robin 1990). Recently, scoliosis has been produced consistently in young chickens following pinealectomy and the curves produced have many similarities to those seen in AIS (Machida *et al.* 1994; Kanemura *et al.* 1997; Wang *et al.* 1997a). This model has been shown to be equally successful in both White Leghorn and Mountain Hubbard chickens (Wang *et al.* 1997, 1998; O'Kelly *et al.* 1999). White Leghorn chickens are raised to produce eggs (Layers) and therefore have been genetically cultivated to mature slowly and start laying eggs approximately 20-25 weeks after hatching. In contrast, Mountain Hubbard chickens are primarily raised to produce meat and have a much higher growth rate, becoming mature nine weeks after hatching. This experiment was designed to examine whether differences in growth rate affect the development of scoliosis following pinealectomy in young chickens.

5.2 METHODS

Eighty, newly-hatched White Leghorn and forty-two newly-hatched Mountain Hubbard chickens were obtained from a local hatchery (Lilydale, Edmonton). They were immediately introduced into a 12:12 light / dark cycle

(Dark 9:00am –9:00 pm), and provided food and water *ad libitum*. The chickens were assigned to the following groups.

Group 1	n = 71	Pinealectomized White Leghorn chickens
Group 2	n = 9	Control White Leghorn chickens
Group 3	n = 35	Pinealectomized Mountain Hubbard chickens
Group 4	n = 7	Control Mountain Hubbard chickens

The surgical procedure was performed between three and five days after hatching. Each chicken underwent the complete removal of the pineal gland (Appendix A). The pineal gland was exposed by making an incision in the skull and the gland was removed with forceps after the stalk had been cut. After surgery the chickens were placed under a heat lamp and observed until they were fully recovered from the surgery. The control chickens did not under any surgical procedure. The chickens were observed for five weeks to determine the incidence and severity of scoliosis, progression of the curve and the growth pattern of each chicken.

Radiography

Supine, anteroposterior radiographs were taken weekly while the chickens were anaesthetized using halothane. To ensure that the spine was extended, small weights (115 g) were attached to each leg and the neck was held extended by the anaesthetic mask. The radiographs were examined for the presence of scoliosis with the degree of curvature was measured using the Cobb technique (Cobb 1960). Angles greater than 10° were defined as scoliosis. Progressive scoliosis was defined as a curve that increased by 4° or greater on each successive week. Differences in the incidence and progression of scoliosis was assessed using Fisher's exact probability test with the level of significance set at $p < 0.05$.

Weight

The weight of each chicken was measured weekly as an indicator of growth and was used to calculate the percentage weight gain during each weekly interval (percent weight gain = {(final weight – initial weight) / initial weight} X 100%) which represented the growth rate. Differences in weight and the average percentage weight gain among experimental groups were assessed using ANOVA followed by Fisher's PLSD ad hoc test with the level of significance set at $p<0.05$.

Spinal Length

The spinal length was measured from the superior border of the body of vertebra T1 to the inferior border of the body of vertebra L2 on each radiograph as an indicator of linear growth. For chickens with scoliosis the spinal length was determined by tracing the path of the curve. Differences in the average spinal length among groups were tested using ANOVA followed by Fisher's PLSD ad hoc test with the level of significance also set at $p<0.05$.

5.3 RESULTS

The surgical procedure appeared to produce no adverse effects on the chickens. On average, chickens took approximately one-hour to recover after which they appeared to feed and behave normally.

The incidence of scoliosis development in pinealectomized chickens is shown in Table 5-1. There was a significantly greater number of Mountain Hubbard chickens (69%) that had developed scoliosis than White Leghorn chickens (39%) two weeks after surgery. The number of Mountain Hubbard chickens that developed scoliosis then remained relatively constant while the number of White Leghorn chickens that developed scoliosis continued to increase. The difference in incidence between the two groups became not significant three weeks after surgery.

A pattern similar to the difference in the incidence of scoliosis is also seen in the severity of scoliosis between the two species (Figure 5-1). Pinealectomized Mountain Hubbard chickens had a significantly more severe deformity on average during the early stages (weeks 2-3), but no significant difference was detected in the 4th week after surgery. In addition, no significant difference was found between the incidence of progressive curves in pinealectomized Mountain Hubbard (20%) and White Leghorn chickens (27%) (data not shown).

The Mountain Hubbard chickens weighed significantly more than White Leghorn chickens for the duration of the experiment (Figure 5-2). In contrast, there were no significant differences in the average weight among the

Table 5-1. The Incidence of Scoliosis in Pinealectomized Mountain Hubbard and White Leghorn Chickens

Group	n	Number (%) of Chickens with Scoliosis		
		Week 2	Week 3	Week 4
Mountain Hubbard	35	69	63	60
White Leghorn	71	39	49	54
p - value		0.004	0.12	0.67

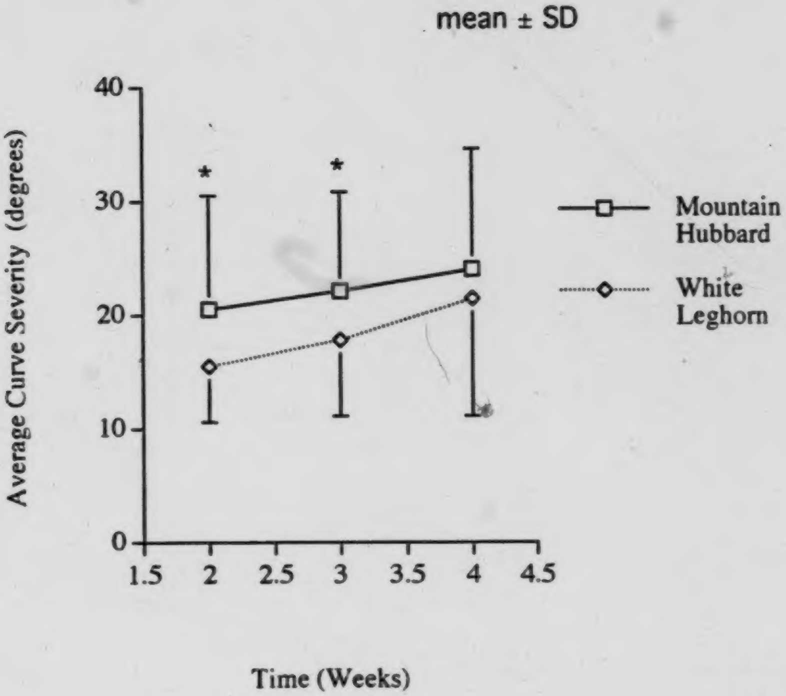

Figure 5-1. The average curve severity of chickens with scoliosis. Mountain Hubbard chickens had a significantly higher curve severity than White leghorn chickens during the second and third weeks after surgery. The difference was not significant in the fourth week. The astericks (*) denotes a significant difference in curve severity between the two groups.

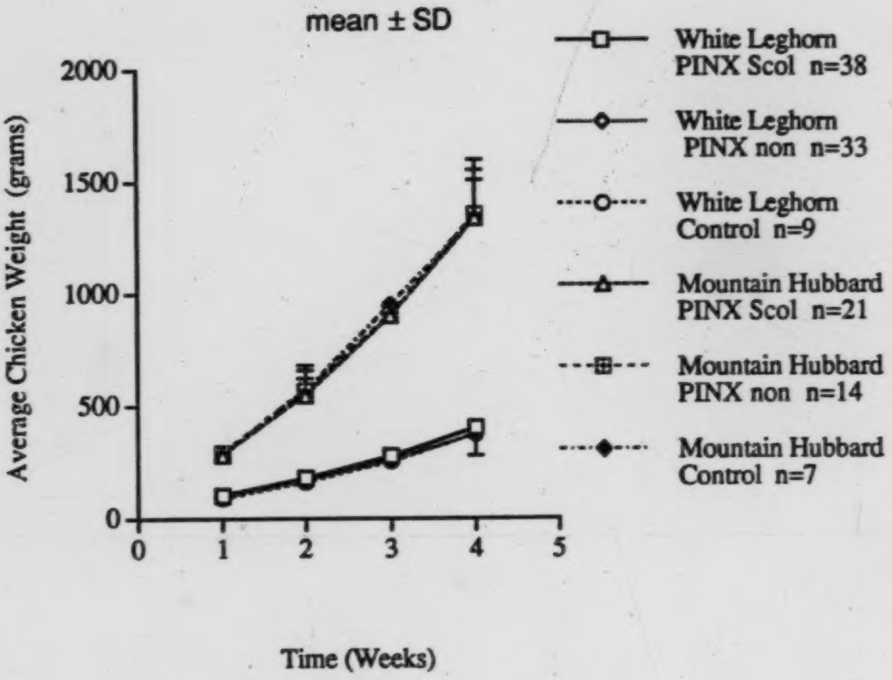

Figure 5-2. Weight of White Leghorn and Mountain Hubbard chickens. No significant differences were found between any of the subgroups within a species. The Mountain Hubbard chickens had a significantly greater weight during the experiment.

PINX Scol = Pinealectomized chickens that developed scoliosis
PINX non = Pinealectomized chickens that did not develop scoliosis

subgroups (Pinealectomy scoliosis, Pinealectomy non-scoliosis, and Control) within either of the species.

The average percentage weight gain per week is shown in Figure 5-3. During the second week the White Leghorn chickens had a significantly lower average percentage growth rate than their Mountain Hubbard counterparts in all groups. During the third week, the pinealectomized White Leghorn chickens as a group had a significantly lower average percentage growth rate than the pinealectomized Mountain Hubbard chickens as a group, whereas the controls did not differ between species. In the final week of comparison only the pinealectomized White Leghorn chickens that did not develop scoliosis had a significantly lower average percent growth rate than the corresponding group of Mountain Hubbard chickens.

There were no significant differences in the percentage growth rates among the subgroups (Pinealectomy scoliosis, Pinealectomy non-scoliosis, and Control) within either of the two species during any time period.

The average spinal length (T1-L2) was significantly greater for Mountain Hubbard chickens compared to White Leghorn chickens throughout the experiment (Figure 5-4) which mirrors the results of body weight. Similarly, no significant differences were detected among the subgroups (Pinealectomy scoliosis, Pinealectomy non-scoliosis, and Control) of a species at any time during the experiment.

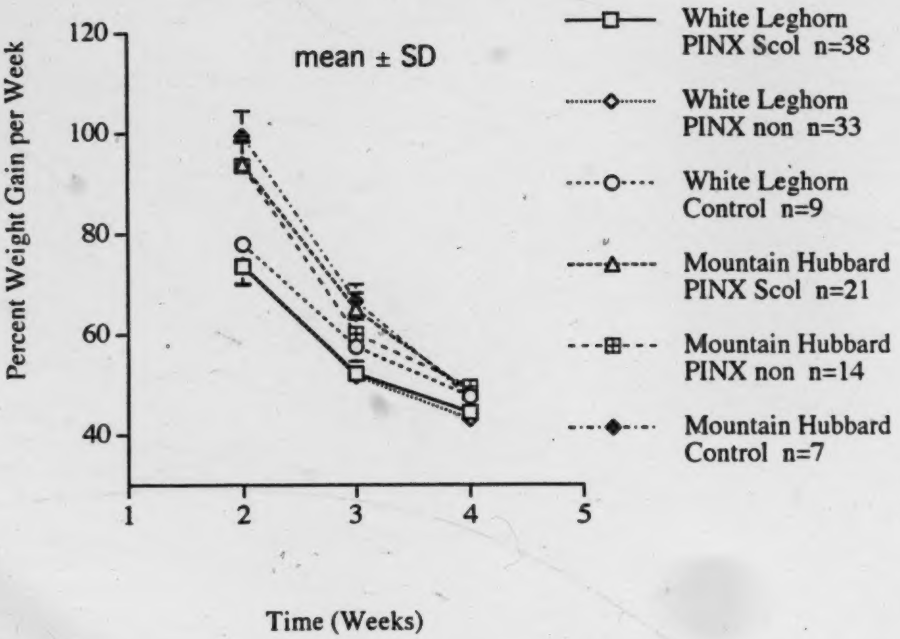

Figure 5-3. Percentage weight gain per week two, three and four weeks after surgery. During week 2 the percentage weight gain of White Leghorn chickens were all significantly lower than their Mountain Hubbard counterparts. During the third week the pinealectomized White Leghorn chickens had a significantly lower percentage growth rate than the pinealectomized Mountain Hubbards. By the fourth week only the pinealectomized White Leghorn chickens that developed scoliosis had a significantly lower percentage weight gain than their Mountain Hubbard counterparts.

PINX Scol = Pinealectomized chickens that developed scoliosis
PINX non = Pinealectomized chickens that did not develop scoliosis

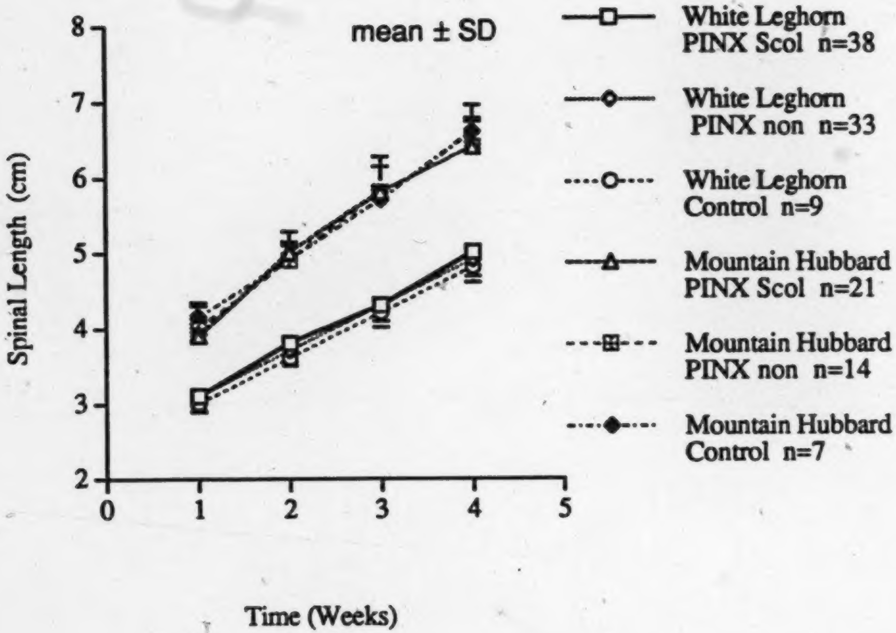

Figure 5-4. Spinal Length of White Leghorn and Mountain Hubbard chickens. No significant differences were found between any of the subgroups within a species. The Mountain Hubbard chickens had a significantly longer spine through out the experiment.

PINX Scol = Pinealectomized chickens that developed scoliosis
PINX non = Pinealectomized chickens that did not develop scoliosis

5.4 DISCUSSION

The results of this experiment have confirmed that Mountain Hubbard chickens grow much faster than White Leghorn chickens during the initial stages of development. Body weight and spine length are always greater in Mountain Hubbards at all stages. The percentage growth rate is initially significantly greater in Mountain Hubbard chickens, but after four weeks the difference in percent growth rate is minimal.

Results also confirm that not all pinealectomized chickens get scoliosis but the percent that ultimately develop scoliosis is similar between the two species. After four weeks the incidence and severity of scoliosis is not significantly different between the two species. However, prior to the fourth week the Mountain Hubbards had a higher incidence and severity of scoliosis than the White Leghorns.

The correlation between higher growth rate and scoliosis development found in this study has also been documented in patients with AIS. Goldberg *et al.* (1993) found that patients with AIS underwent their maximal growth phase earlier than individuals without scoliosis. Similarly, Ahl *et al.* (1988) found that patients with AIS had elevated serum growth hormone concentrations compared to controls. Furthermore, GH treatment in humans has been reported to increase the severity of scoliosis (Dymling and Willner 1978). Similarly, this study has identified that Mountain Hubbard chickens develop scoliosis earlier than White Leghorns but the difference is eliminated as growth continues. This confirms that growth is an important aspect of scoliosis development in pinealectomized chickens similar to that in humans.

The incidence of scoliosis was found to decrease slightly in Mountain Hubbard chickens after the second week. This decrease is attributed to initially mild curves (10° -15°) dropping below 10° and were no longer defined as scoliosis.

It would be interesting to see if reduced levels of growth hinder scoliosis development in chickens. Wang *et al.* (1997b) did a preliminary study of the development of scoliosis in older chickens which have reduced growth rates and found a reduced incidence and severity of scoliosis which supports the relationship between growth rate and scoliosis development. However, the reduced levels of scoliosis may also have been due to the ossification of spinal ligaments that occurs in older chickens (Jiang *et al.* 1994) which may hinder curve development.

In conclusion, Mountain Hubbard chickens have a higher percentage growth rate during the early stages of development compared to White Leghorn chickens. In addition, the incidence and severity of the curve is also greater in Mountain Hubbard during these early stages. These results indicate a relationship between the percentage growth rate and curve development in pinealectomized chickens.

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Chapter 6

The Influence of Gender on the Development of Scoliosis in Pinealectomized Chickens.

6.1 INTRODUCTION

Adolescent idiopathic scoliosis (AIS) is a three dimensional deformity characterized by lateral curvature of the spine and vertebral rotation (Lonstein 1994). The consequences of such a deformity are considerable but despite extensive research the cause remains unknown. One of the factors limiting research in this area has been the lack of an appropriate animal model (Robin 1990). However, scoliosis that closely resembles AIS (Machida *et al.* 1994; Kanemura *et al.* 1997; Wang *et al.* 1997) has recently been shown to be consistently produced in young chickens following pinealectomy (Machida *et al.* 1993, 1994, 1995, 1997; Coillard & Rivard 1996; Kanemura *et al.* 1997; Wang *et al.* 1997, 1998; O'Kelly *et al.* 1999). This model is exciting because it has the possibility to provide further insights into scoliosis and result in novel treatment options.

AIS is reported to affect mainly females (Lonstein, 1994) with estimates of 80% being common. Although it has received considerable attention, this aspect of AIS has never been satisfactorily explained. Considering its significance it would be interesting to determine whether this pattern was duplicated in the chicken model. Several authors have used a combination of both sexes of chickens but have not explored the influence of gender (Thillard 1959; Machida *et al.* 1993, 1994, 1995; Coillard & Rivard 1996; Wang 1997, 1998). Other studies have specifically used only one gender and have reported incidences of 100% in males (Machida *et al.* 1997) and 68% in females (Kanemura *et al.* 1997) but it is difficult to compare these results directly because of the different methodologies employed. Therefore the objective of this study was to determine if gender had any effect on the development of scoliosis in pinealectomized chickens similar to that observed in AIS.

6.2 METHODS

Ninety-three, newly-hatched chickens were obtained from a local hatchery (Lilydale, Edmonton). Their gender was professionally identified by the hatchery based on wing feather length. This identification was later confirmed by comb development. The chickens were immediately transferred to a single pen with a 12:12 light / dark cycle (Dark 9:00am –9:00 pm), and provided food and water *ad libitum*. The chickens were assigned to the following groups.

Group 1	n = 37	Female Pinealectomy
Group 2	n = 34	Male Pinealectomy
Group 3	n = 16	Male Control
Group 4	n = 6	Female Control

There was a shortage of female chickens, and a decision was made to maximize the number of female pinealectomized chickens so that valid comparisons could be made between the scoliosis and non-scoliosis subgroups. Unfortunately this occurred at the expense of not having many control female chickens.

The surgical procedure was performed between three and five days after hatching. Groups 1 and 2 (Pinealectomy) underwent the complete removal of the pineal gland (Appendix A). The pineal gland was exposed by making an incision in the skull and the gland was removed with forceps after the stalk had been cut. Group 3 and 4 (Control) did not undergo any surgical procedure.

After surgery the chickens were placed under a heat lamp and observed until they were fully recovered from the surgery. These chickens were observed for five weeks to determine the incidence and severity of scoliosis, curve pattern and the serum melatonin levels in each group.

Radiography

Supine, anteroposterior radiographs were taken weekly while the chickens were anaesthetized using halothane. To ensure that the spine was extended small weights (115 g) were attached to each leg and the chicken's neck was held extended by the anaesthetic mask. A previous study has found that the radiographic technique and curve measurements were reproducable to within ± 3 degrees (Bagnall *et al.* Unpublished). The radiographs were examined for the presence of scoliosis with the degree of curvature assessed using the Cobb technique (Cobb 1960). Angles greater than 10° were defined as scoliosis. Progressive scoliosis was defined as a curve that increased by 4° or greater on each successive week. In addition the curve pattern, either a double or single curve, was observed on the radiographs. Differences in the incidence and pattern of scoliosis was assessed using Fisher's exact probability test, with the level of significance set at $p < 0.05$.

Blood Collection

Blood samples were collected in the middle of the dark cycle from a wing vein using a non-heparinized #18-gauge needle. The only sources of light were subdued red lights with an average intensity of 10 lux. These lighting conditions do not effect the level of serum melatonin in chickens (Reiter 1991, Chapter 7). The time needed to complete blood collection for all 87 chickens was less than two hours. The blood was left at room temperature for four hours to allow for coagulation to occur and stored 16-24 hours at 4° C. The blood samples were cryo-centrifuged for 20 minutes at 3000 rpm at 4° C and the serum stored at –20° C until assayed within two weeks.

Melatonin Assay

The amount of melatonin present in the serum was assessed using a competitive binding ^{3}H-melatonin assay (Appendix B). Each sample was assayed in duplicate and the average value was used for the analysis.

Differences in the average serum melatonin levels between the four groups was assessed using analysis of variance (ANOVA) followed by Fisher's PLSD ad hoc test with the level of significance set at $p < 0.05$.

Weight

The weight of each chicken was measured weekly and used to calculate the percentage weight gain during each weekly interval (% weight gain = {(Weight 2 – Weight 1)/Weight 1} X100%). Differences in the percentage weight gain among experimental groups were assessed using ANOVA followed by Fisher's PLSD ad hoc test ($p<0.05$).

6.3 RESULTS

The surgical procedure appeared to produce no adverse effects on the chickens. On average, chickens took approximately one-hour to recover, after which time they appeared to feed and behave normally.

The pattern of scoliosis development is shown in Table 6-1. The control groups are not included because none of the chickens in the control groups developed scoliosis during the experiment. No significant difference in the incidence of scoliosis was found between male and female pinealectomized chickens during the five week experiment. The final percentage of male pinealectomized chickens that developed scoliosis was

Table 6-1 The Incidence of Scoliosis in Male and Female Pinealectomized Chickens

Group	n	Number (%) of Chickens with Scoliosis			
		Week 1	Week 2	Week 3	Week 4
Male	34	35%	44%	56%	59%
Female	37	43%	51%	51%	54%
p-value		0.4745	0.6126	0.6956	0.3798

59% (20/34) compared to 54% (20/37) of the female pinealectomized chickens.

Thirty percent (6/20) of pinealectomized males were considered to have progressive curves, compared to twenty-five percent (5/20) of the pinealectomized females. This difference was found to be non-significant by Fisher's exact probability test.

The average severity of scoliosis for male and female pinealectomized chickens is shown in Figure 6-1. The curve severity in the female group (27° ±14°) is consistently higher compared to the males (22° ± 10°) after five weeks. However, these differences were not significant.

A significant difference was found between the proportion of males (1/20) and females (7/20) that developed double curves. This was the only criteria by which the scoliosis differed between the male and female pinealectomized chickens.

The serum melatonin levels during the middle of the dark cycle on the third through fifth weeks after surgery are shown in Figure 6-2. The average serum melatonin levels of the pinealectomy groups are all significantly lower than the average serum melatonin levels of the control group. In addition the serum melatonin levels for the pinealectomized chickens that developed scoliosis were not significantly different from those pinealectomized chickens that did not develop scoliosis and the serum melatonin levels for the males did not differ significantly from the females. The patterns are consistent for week 3, week 4, and week 5 (Figure 6-2).

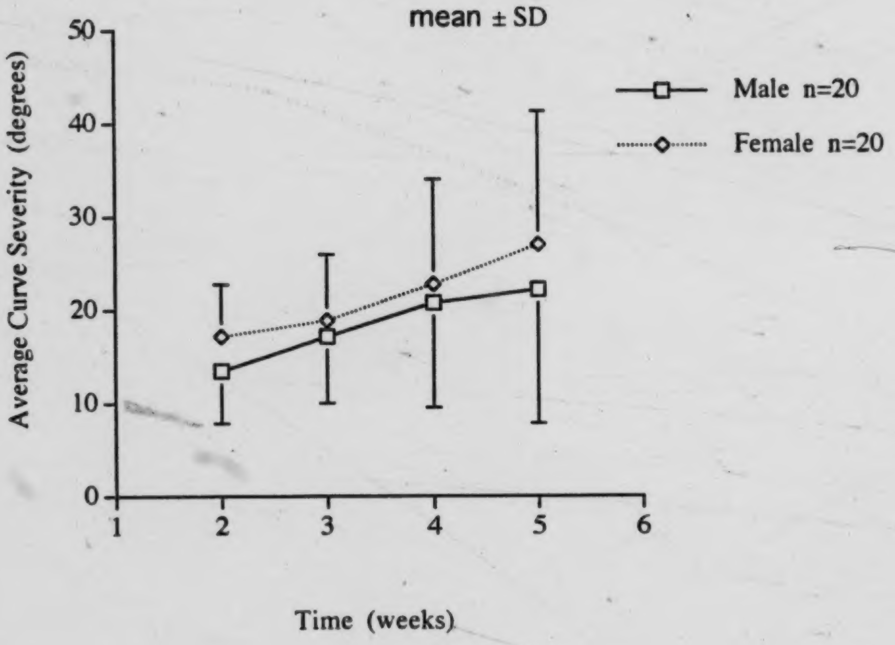
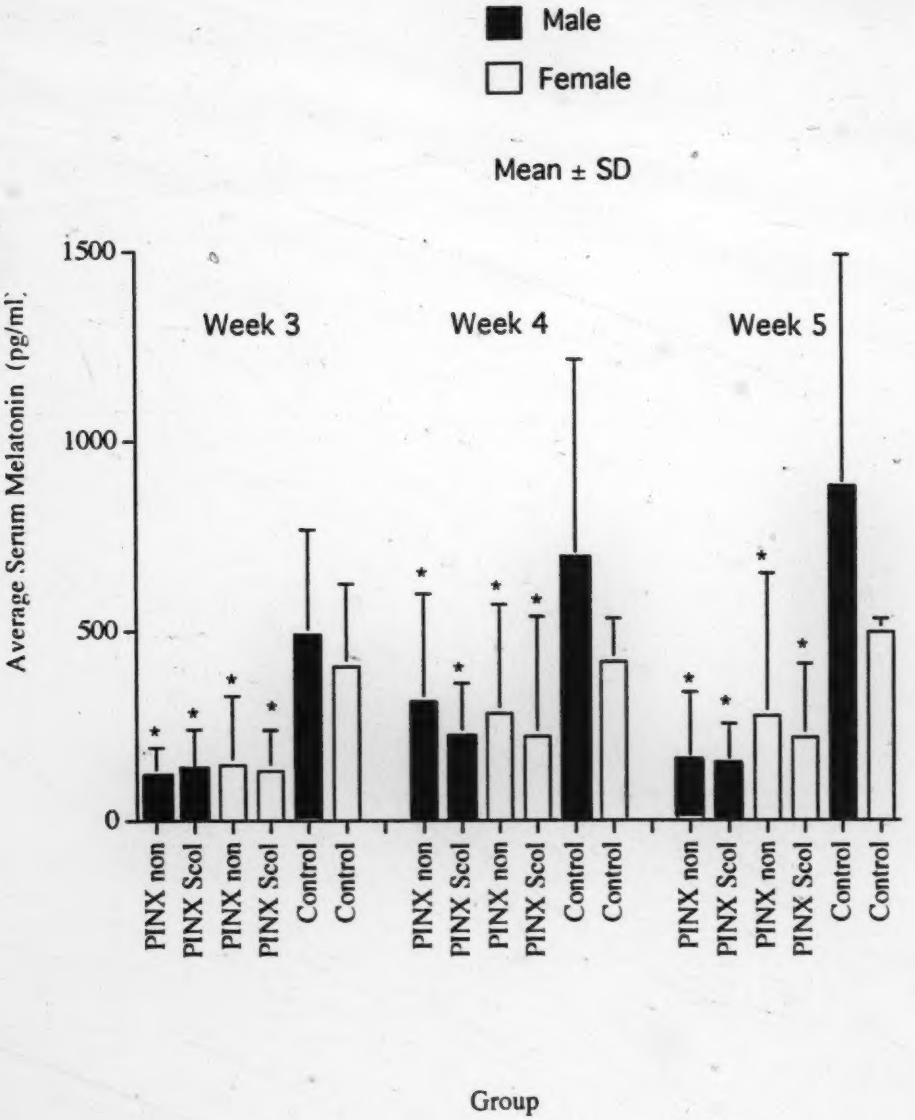


Figure 6–1. The mean curve severity for male and female pinealectomized chickens during weeks two – five. The average curve severity did not differ between males and females during any time during the experiment.

Figure 6-2. The mean night-time serum melatonin levels for chickens three, four and five weeks following pinealectomy surgery compared to controls. No significant differences were found among any of the pinealectomized groups during the experiment. However, the serum melatonin levels of the pinealectomized groups were significantly lower than the controls within each gender each week. The asterisk (*) denotes a statistically significant difference ($p<0.05$) from the control group within each gender.

Male PINX non = Male pinealectomy group that did not develop scoliosis. n=14
Male PINX Scol = Male pinealectomy group that developed scoliosis. n=20
Female PINX non = Female pinealectomy group that did not develop scoliosis. n=17
Female PINX Scol = Female pinealectomy group that developed scoliosis. n=20
Male Control. n=16
Female Control. n=6


■ Male
□ Female
Mean ± SD
Average Serum Melatonin (pg/ml)
1500
1000
500
0
Week 3
Week 4
Week 5
*
PINX non
PINX Scol
PINX non
PINX Scol
Control
Control
Group

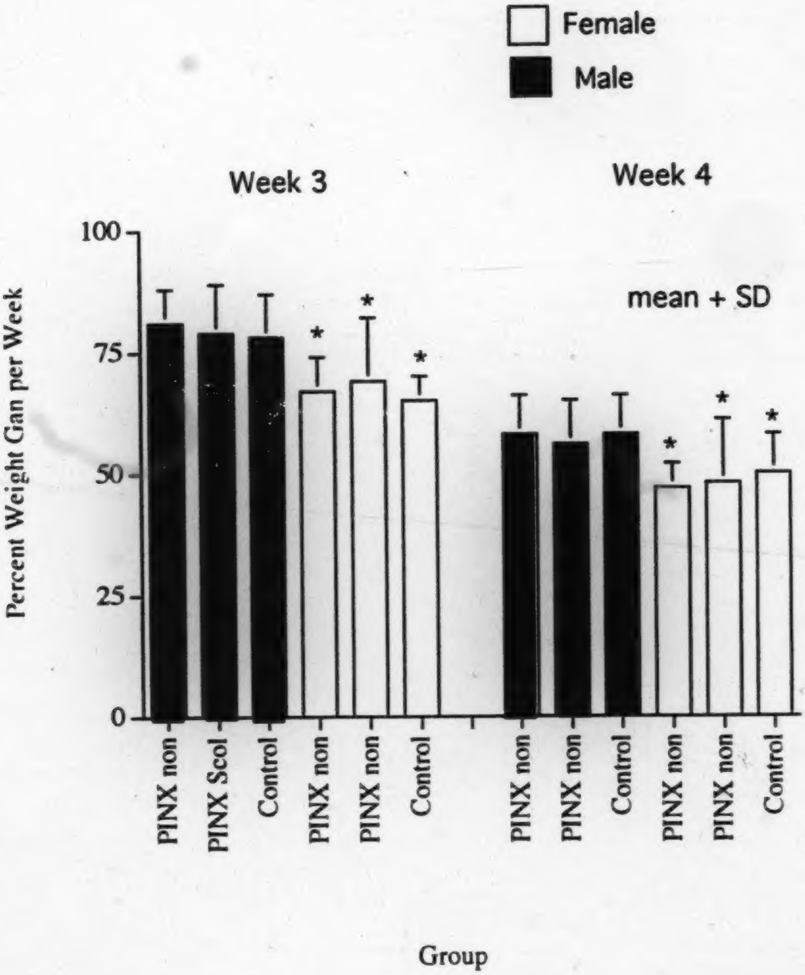
The percentage weight gain per week is represented in Figure 6-3. For both the second and third weeks there were no significant differences between the pinealectomized and control chickens within each gender. Furthermore, the percentage weight gain per week did not differ between those pinealectomized chickens that developed scoliosis and those that did not. However a consistent pattern was seen with the female chickens having a significantly lower percentage growth rate compared to the male chickens.

6.4 DISCUSSION

The results of this study indicate that the incidence, average curvature, and proportion of curves that are considered progressive do not differ between male and female pinealectomized chickens. The only difference in the pattern of scoliosis development between males and females is that females tend to develop more double curves. In AIS, double curves more commonly associated with curve progression (Lonstein 1994). Since the proportion of females with double curves was greater than males with double curves it was suspected that females would also show a greater tendency to have progressive curves. However the results of this study do not support that hypothesis. This may be due to differences in the factors leading to progression in pinealectomized chickens compared to patients with AIS, but may also be due to differences in the definition of progression between pinealectomized chickens and patients with AIS. In AIS, progressive scoliosis

Figure 6-3. The percentage weight gain per week of chickens three and four weeks following pinealectomy surgery. The asterisk (*) denotes a statistically significant difference ($p<0.05$) from the control group within each gender.

Male PINX non = Male pinealectomy group that did not develop scoliosis. n=14
Male PINX Scol = Male pinealectomy group that developed scoliosis. n=20
Male Control. n=16
Female PINX non = Female pinealectomy group that did not develop scoliosis. n=17
Female PINX Scol = Female pinealectomy group that developed scoliosis. n=20
Female Control. n=6


Female
Male
Week 3
Week 4
mean + SD
100
75
50
25
0
Percent Weight Gan per Week
PINX non
PINX Scol
Control
PINX non
PINX non
Control
PINX non
PINX non
Control
PINX non
PINX non
Control
*
Group

is generally defined as a curve that increased by >10° on radiographic examination over a six month period. Clearly the same definition cannot be used for pinealectomized chickens. For this study an increase of 4° per week was defined as a progressive curve. Larger increases (i.e 5°-7°) were not chosen as the definition of progression in a desire reduce the number of false negatives, and smaller values (2°-3°) were below the level of accuracy of the measuring procedure. It is possible that a difference in the incidence of progression existed between males and females but our procedure was unable to detect it.

The average serum melatonin levels for pinealectomized chickens that develop scoliosis were not significantly different from those that did not develop scoliosis. This confirms other studies that have found that serum melatonin levels are a poor predictor of scoliosis development in pinealectomized chickens (Wang *et al.* 1998; Chapters 3, 4). Furthermore, no difference in average serum melatonin levels were found between the male and female pinealectomized chickens during this experiment. Therefore serum melatonin levels cannot be used to explain the higher incidence of double curves in the females compared to the male pinealectomized chickens.

The only other gender difference in pinealectomized chickens appears to be the percentage growth rate with females growing at a lesser rate during the first four weeks. That may indicate that females have lower growth hormone levels than males which is interesting since growth hormone was found to be associated with scoliosis development in humans (Ahl *et al.* 1988). These data indicate that it may be involved in double curve development in pinealectomized chickens. However, scoliosis in humans is associated with high growth hormone levels (Ahl *et al.* 1988) whereas the females in this study presumably have low growth hormone levels as indicated by their low growth rate.

There are two pieces of evidence to support the claim that a low growth rate is not directly linked to scoliosis development in pinealectomized chickens. First, the growth rate did not differ between female chickens that developed scoliosis and those that did not. Second, the incidence of scoliosis did not differ between male and female pinealectomized chickens even though the growth rate differed between those two groups. However, a low growth rate may be related to the high incidence of double curves in females.

The results of this experiment support the concept that there are two phases in the development of scoliosis. The first phase that is associated with the initial development of the curve and the second phase that determines how the curve will develop (Lonstein 1994). It appears that gender does not influence the phase of scoliosis initiation because the incidence of scoliosis does not differ between males and females. However differences in the proportion of double curves between males and females may indicate that gender influences the pattern of curve development in pinealectomized chickens.

Despite the similarities notes in this experiment, there are limitations in the ability to directly compare the effect of gender between humans and chickens. There are vast physiological, endocrinological, anatomical, and genetic differences between the two species which will affect the role of gender. For example AIS develops during the period of sexual maturation in humans which is accompanied by hormonal changes. However, scoliosis develops in pinealectomized chickens well before they reach sexual maturation. As a result the hormonal effect of gender may not be comparable between the species.

In conclusion female pinealectomized chickens tend to develop more double curves than male pinealectomized chickens. In addition, females have a lower growth rate than their male counterparts. However, definitive evidence that these two phenomena are related has yet to be shown.

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Chapter 7

The Immediate Effect of Light on Serum Melatonin Levels During the Dark Cycle in Young Chickens.

7.1 INTRODUCTION

Adolescent idiopathic scoliosis (AIS) is a three dimensional deformity characterized by a lateral curvature of the spine including vertebral rotation and rib cage deformity (Lonstein 1994). Despite extensive research there is no known cause and investigation has been hampered by the lack of an appropriate animal model (Robin, 1990). In recent years, pinealectomy in young chickens has consistently resulted in the development of scoliosis that has many characteristics similar to those seen in patients with AIS (Machida *et al.* 1994; Kanemura *et al.* 1997; Wang *et al.* 1997). Determination of the mechanism underlying this animal model may provide clues to the etiology of AIS and possibly lead to improvements in clinical management. Unfortunately, the mechanism underlying the development of scoliosis following pinealectomy in young chickens is unknown and its relevance to AIS remains to be determined.

Melatonin is the principal product of the pineal gland (Reiter 1991) and has been the logical focus of many studies investigating this phenomenon. However, results from these studies are inconsistent, with the results from some studies supporting the role of melatonin (Machida *et al.*, 1993, 1995, 1997) while the results from others have been less supportive (Bagnall *et al.*

1999; Wang *et al.*, 1998; Chapter 4). Melatonin secretion from the pineal gland occurs during the dark phase of a 24 hour light-dark cycle (Bagnall *et al.* 1999) but its secretion can be affected by environmental lighting conditions (Reiter, 1991). Consequently, attempts to explain inconsistencies in the serum melatonin studies involving pinealectomized chickens have included questions related to lighting conditions during the blood sample collection. While it is well established that interruption of the dark phase with prolonged light exposure leads to decreasing melatonin levels in some mammals (Reiter, 1991), the effects of short periods of illumination, which are necessary for blood collection in chickens, have not been investigated.

Accordingly, this experiment was designed to determine the immediate changes in serum melatonin levels in the chicken following acute exposure to white and red light during the middle of the dark cycle. The results of this experiment will provide guidance with regard to lighting conditions needed to ensure valid and reliable measurements of serum melatonin levels in chickens.

7.2 METHODS

In all experiments, newly-hatched Mountain Hubbard chickens were obtained from a local hatchery (Lilydale, Edmonton). The chickens were immediately introduced to a 12:12 light-dark cycle (4:30 a.m.- 4:30 p.m. dark) and kept on this cycle throughout the experiment. The chickens were kept in a single pen with constant environmental conditions (26°C and 70% relative humidity) and were fed food and water *ad libitum*.

Experiment 1 This experiment was conducted as a pilot study to determine the general effect of white light exposure on the serum melatonin levels of chickens during the middle of the dark cycle. To measure this effect serum melatonin levels were determined in fourteen, five week old chickens in each of three different lighting conditions:

Condition 1: Blood samples were collected during the dark in the middle of the dark cycle. These chickens acted as a control group (n=14).

Immediately following blood collection, the white lights (750 Lux) were turned on.

Condition 2: Blood samples were collected after 30 minutes exposure to the white light. (n=14)
Condition 3: Blood samples were collected after 60 minutes exposure to the white light. (n=14)

Experiment 2 This experiment was designed to measure the effect of introducing white light in the middle of the dark cycle on the serum melatonin levels of young chickens and the effect of reintroducing darkness following exposure to the white light. Five days after hatching, one hundred chickens were randomly assigned to the following groups:

Group 1: A normal control group that had blood collected in the dark (3 Lux) before white light was introduced (n=10).

Group 2: (9:15 a.m.) - blood collected immediately after turning on white light (750 Lux) (n=10).

Group 3: Blood collected after 10 minutes of exposure to white light (n=10).

Group 4: Blood collected after 20 minutes of exposure to white light (n=10).

Group 5: Blood collected after 30 minutes of exposure to white light (n=10).

After group 5 was completed the white lights were turned off and 3 groups of chickens were transferred immediately to a dark, light-proof box and another group remained exposed to red light. All subsequent blood collection occurred in red lighting.

Group 6: Blood collected 10 minutes after the chickens were re-introduced to darkness (n=10).

Group 7: Blood collected 20 minutes after the chickens were re-introduced to darkness (n=10).

Group 8: Blood collected 30 minutes after the chickens were re-introduced to darkness (n=10).

Group 9: Blood collected from the group that had been exposed to red light for 30 min. (n=10).

Group 10: A normal control group that had remained in a dark, light-proof box for the duration of the experiment (n=10). This group acted as a control for comparison with the values from group 1.

Experiment 3 This experiment was designed to determine the effects of exposure to red light during the middle of the dark phase on serum melatonin levels in young chickens. The effects of subsequently introducing white light were also examined. One hundred and twenty, 5-day old chickens were randomly assigned to one of the following groups:

Group 1: (9:15a.m.) - blood collected immediately as red lights were turned on (3 Lux) (n=10).

Group 2: Blood collected after 10 minutes of exposure to red light (n=10).

Group 3: Blood collected after 20 minutes of exposure to red light (n=10).

Group 4: Blood collected after 30 minutes of exposure to red light (n=10).

Group 5: Blood collected after 40 minutes of exposure to red light (n=10).

Group 6: Blood collected after 50 minutes of exposure to red light (n=10).

Group 7: Blood collected after 60 minutes of exposure to red light (n=10).

Group 8: A normal control group that had been maintained in total darkness. This group acted as a control group for group 1 (n=10).

After group 8 was completed, white light (750 Lux) was introduced and blood collection continued as follows.

Group 9: Blood collected immediately upon exposure to white light (n=10).

Group 10: Blood collected following 10 minutes of exposure to white light (n=10).

Group 11: Blood collected following 20 minutes of exposure to white light (n=10).

Group 12: Blood collected following 30 minutes of exposure to white light (n=10).

Blood Collection

In experiment 1 the chickens were five weeks old, at which time they were large enough to survive the loss of 3 ml of blood. This allowed for the same chicken to be sampled repeatedly in each of the three conditions. One ml of blood was withdrawn during each time period from a wing vein using a #18-gauge needle and immediately transferred gently to a non-heparinized, glass tube to avoid cell damage. The blood was left at room temperature for two hours prior to being stored at 4°C for 24 hours to allow for coagulation to take place.

The collection of blood in experiments 2 and 3 consisted of injecting the chicken with an overdose of euthanyl, followed immediately by opening the chest cavity and withdrawing 1 ml of blood from the heart using a #18-gauge needle. This was necessary because the amount of blood needed for the assay could not be satisfactorily obtained from the wing vein, and the chickens were not sufficiently large to survive the loss of 1 ml of blood at five days of age. After the blood was collected it was treated as described previously.

When blood samples were collected 'in the dark', red lighting was actually used but only while the samples were being collected because some light was needed for safe manipulation of the instruments.

Serum Melatonin Assay

The serum melatonin levels of each sample were measured in duplicate (average value used for the analysis) using a competitive binding radioimmunoassay used successfully in a previous study (Bagnall *et al.* 1996) and described in Appendix B.

Light Conditions

Light intensity was measured with a lux-meter and the average intensity was calculated using values from each of the four corners and the middle point of the table that was used in the experiment. White light was obtained from overhead fluorescent lights and white bulbs (60 Watt) inserted into three ordinary table lamps that were distributed around the table. Red light was obtained from red bulbs (40 Watt) inserted into three ordinary table lamps and measured an average of 4 Lux.

In all experiments, the mean serum melatonin levels between groups at each of the time intervals were compared using ANOVA followed by Fisher's PLSD ad hoc test with the level of significance was set at $p<0.05$.

7.3 RESULTS

Blood collection was simple and took approximately 6 minutes for each group of 10 chickens. However, in some instances insufficient blood was collected usually because the heart had stopped beating prior to the insertion of the needle into the heart. This resulted in some groups having fewer samples for final analysis than those indicated in the methods section.

Experiment 1 (Pilot study)

The results from the pilot study are shown in Figure 7-1. The average serum melatonin levels collected 30 minutes (57 ± 35 pg/ml) and 60 minutes (57 ± 54 pg/ml) after the introduction of white light were both significantly lower than the control values taken from blood samples collected in the dark (295 ± 293 pg/ml). Furthermore the average serum melatonin levels 30 minutes following the introduction of white light were not significantly different from those collected after 60 minutes.

Experiment 2

The results of experiment 2 are summarized in Figure 7-2. The serum melatonin levels became significantly lower than the control value (group 1)

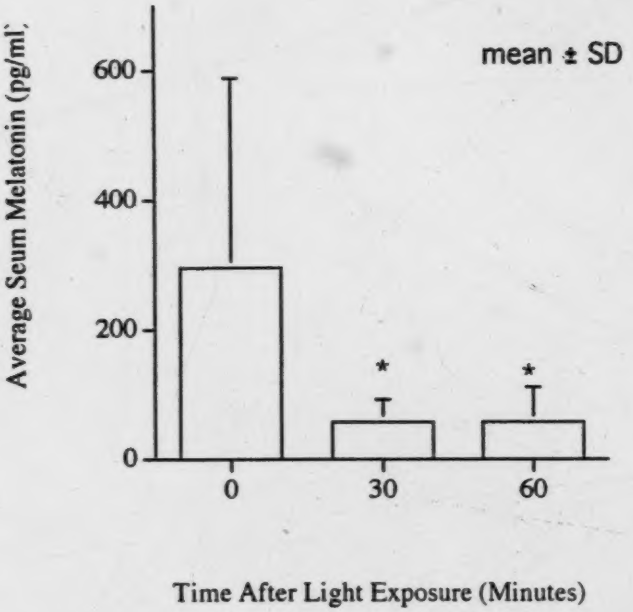

Figure 7-1. Average serum melatonin levels of chickens during the peak phase of melatonin secretion and following exposure to white light (~750 lux). A significant decrease in serum melatonin is noticed within 30 minutes. An asterisk (*) denotes a significant difference compared to t=0.

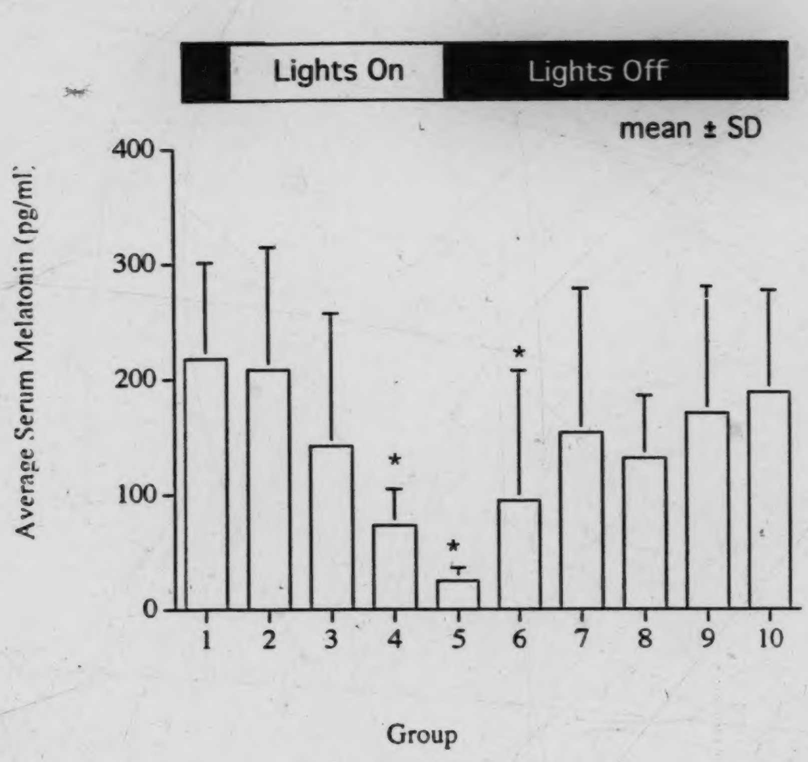

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Figure 7-2. Average serum melatonin levels of chickens in the middle of the dark cycle following exposure to white light followed by re-introduction of darkness. Each group is separated by 10 minutes. The serum melatonin levels become significantly reduced after exposure to white light for 20 minutes (Group 4) but regained normal levels within 20 minutes after the re-introduction of darkness (Group 7). An asterisk (*) denotes a statistically significant difference from group 1.

Group 1: peak serum melatonin level during the middle of the dark cycle
Groups 2-5: Were exposed to white light.
Groups 6-9: White lights were extinguished.
Group 10: Chickens that had been in complete darkness during the entire experiment.

but only after 20 minutes exposure to white light (group 4). The values continued to decrease until the white lights were extinguished, reaching a minimum value after thirty minutes exposure to white light (group 5). When the white light was removed the average serum melatonin levels began to rise and became significantly higher than the minimum level after twenty minutes (group 7). At this time the average serum melatonin levels were no longer significantly different than the original, control levels of group 1. The average serum melatonin levels of the chickens that were sequestered in the light-proof box (group 10) were also not significantly different than the original, control levels (group 1) or from the average serum melatonin levels of the chickens that were re-introduced into darkness for 20, 30, or 40 minutes (groups 7, 8, and 9 respectively).

Experiment 3

The results from experiment 3 are presented in Figure 7-3. The average control values from blood samples collected in the dark (group 1) (411 ± 344 pg/ml) did not differ significantly from any of the other average values collected at ten minute intervals over the next 60 minutes (407 ± 105 , 298 ± 147, 345 ± 148, 390 ± 244, 390 ± 133, 338 ±103 pg/ml respectively) while the chickens were in red light. These values also did not differ significantly from the values obtained from the group of chickens that had been kept in the dark and from which blood had been collected 70 minutes after the experiment started (429 ± 180 pg/ml). When the white lights were

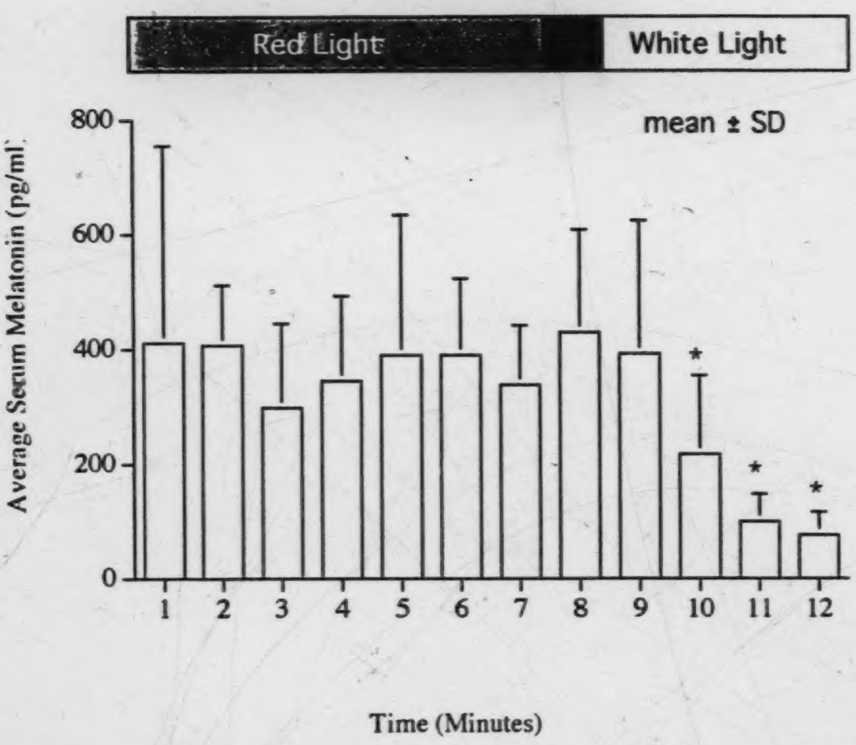

Figure 7-3. The average serum melatonin levels of chickens exposed to a sequence of red and white light during the peak phase of melatonin secretion. Each group is separated by 10 minutes. Maintaining the chickens in red light for 60 minutes does not significantly reduce the serum melatonin levels from the peak values attained in Group 1. Subsequent exposure to white light (Groups 9-12) significantly reduces the serum melatonin from peak levels after only 10 minutes (Group 10). Statistically significant differences are denoted with an asterisk (*).

Group 1: peak serum melatonin level during the middle of the dark cycle.
Groups 2-7: Chickens were exposed to red light.
Group 8: Serum melatonin level of a group of chickens that had remined in the dark for the duration of the experiment.
Groups 9-12: Chickens exposed to white light.

turned on after 80 minutes of exposure to red light, the average serum melatonin level (392 ± 232 pg/ml) became significantly lower than all the previous values after only 10 minutes (217 ± 137 pg/ml) and then continued to decrease 20 minutes (100 ± 48 pg/ml) and 30 minutes (76 ± 40 pg/ml) after white light introduction.

7.4 DISCUSSION

The results from each of the three experiments of this study have confirmed the results of earlier studies in mammals (Reiter 1991; Nozaki *et al* 1990) that the introduction of white light during the middle of the dark cycle significantly reduces serum melatonin levels. However, the results also showed that this reduction occurred after only 10 minutes and reached a minimum after 30 minutes of light exposure. The results also confirmed that red light exposure for up to 60 minutes had no effect on serum melatonin levels. This is relevant to the experimental conditions needed for the collection of blood samples that are to be used for the measurement of serum melatonin. Blood samples from chickens must be collected within the first few minutes after the introduction of white light. Better still, samples should be collected in subdued red light to ensure that the values of serum melatonin are valid and not significantly different from samples collected in the dark.

The production of melatonin is regulated by output from light receptor cells in the retina. Fibres from these cells innervate the pinealocytes following the sympathetic pathway from the superior cervical ganglion and inhibit melatonin production in both mammalian and avian species (Volrath 1981). During darkness this inhibition is released and a cascade of reactions translates tryptophan into melatonin within the pineal gland. Secretion of melatonin into the blood circulation is immediate with no significant amounts being stored in the pineal gland. Circulating melatonin is deactivated by the liver with approximately 90% being removed on the first passage through the liver (Reiter, 1991). Consequently, the half-life of melatonin in the blood is in the range of 15-30 minutes depending on species, size and age (Vaughan *et al.* 1988). The introduction of light in the middle of the dark phase immediately interrupts the pathway of melatonin production at its source, but events already in progress are presumably not affected. Furthermore, although melatonin is produced elsewhere, such as in the retina and wall of the intestine, these amounts are not significant and do not contribute to circulating levels (Weichmann, 1986).

Therefore, melatonin production will cease quickly with the introduction of white light with subsequent rapid decrease in serum melatonin levels as melatonin is removed from the blood without replenishment. The results of this study support this model. However, it must be emphasized that serum melatonin levels remained at valid levels for a short period of time after the exposure to white light presumably because production and secretion were not stopped immediately and circulating melatonin had not yet been removed. This short period of time is when blood can be collected in white light to ensure valid measurements of serum melatonin but it must be emphasized that this window of time is only a few minutes.

It has been shown previously that the wavelength of light affects melatonin production with white light reducing levels close to zero, blue and green reducing levels significantly but not as effectively as white light, and red light having little or no effect (Volrath, 1981). Presumably the receptor cells are sensitive to only a small window of electromagnetic spectrum and that the wavelength of red light lies outside of the window. The results of this experiment have confirmed that white light reduces serum melatonin levels to close to zero in about thirty minutes and that red light appears to have no effect even after 60 minutes of exposure. Consequently, blood samples should be collected in red light which provides sufficient illumination for the manipulation of instruments and also allows valid levels of serum melatonin to be obtained.

While the results of this study are pertinent to the measurement of valid serum melatonin levels in young chickens and exploration of the pinealectomy model for scoliosis in chickens, their relevance to humans must also be considered. This study suggests that the results from animal experiments in which blood samples were collected in white light and which have not included appropriate control groups for comparison must be viewed with caution. The results also emphasize that care must be taken when collecting blood samples from patients with AIS. It is easier to obtain blood samples from patients in the dark simply by using blindfolds to cover their eyes in conjunction with subdued lighting but it must also be realized that inadvertent light might sufficiently affect serum melatonin levels. Further studies need to be undertaken to determine the precise arrangements necessary to ensure valid measurements in humans similar to those demonstrated in this study for chickens.

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Chapter 8

General Discussion and Conclusions

8.1 GENERAL

Despite extensive research in many areas there is no known cause for AIS. In addition, the lack of a widely accepted animal model for the study of AIS has hampered research. Lateral curves of the spine have been produced in several animals using many techniques but none has closely resembled AIS (Robin, 1990). Human studies have been able to determine that curves tend to develop during the rapid period of growth (Goldberg *et al.* 1993), females tend to develop more severe and progressive curves than males (Shohat *et al.* 1988) and females with progressive curves have lower levels of serum melatonin than non-progressive patients or controls (Machida *et al.* 1996).

8.2 PINEALECTOMIZED CHICKENS

The scoliosis that develops in pinealectomized chickens has several characteristics similar to AIS (Machida *et al.* 1994; Kanemura *et al.* 1997; Wang *et al.* 1997). These similarities include the type (single vs. double, right vs. left), severity, the presence of vertebral rotation, and progression. Differences between the two include the fact that vertebrae in chickens wedge prior to curve development whereas wedging occurs after curve development in AIS (Bagnall *et al.* In Press).

The cause of the scoliosis in chickens remains unknown. Scoliosis has been proposed to result from lack of melatonin, but melatonin therapy results have been inconclusive. Melatonin therapy in pinealectomized chickens has been found to decrease the incidence and severity of scoliosis in pinealectomized chickens (Machida *et al* 1995). However, in a similar study, Bagnall *et al.* (1999) found melatonin therapy to be ineffective in altering the development of scoliosis. Alternatively, the scoliosis has been proposed to result from an artifact of the extensive surgery. Definitive evidence to support either hypothesis has yet to be collected.

8.3 QUESTIONS TO ANSWER

1. Does the scoliosis in pinealectomized chickens result from the removal of the pineal gland or another aspect of the surgical procedure?
2. Does scoliosis in pinealectomized chickens result from low levels of serum melatonin?
3 What is the relevance of this model to AIS?

8.4 SUMMARY OF EXPERIMENTAL RESULTS

Chapter 3: Investigating the Possible Role of an Artifact of the Surgical Procedure in the Development of Scoliosis in Pinealectomized Chickens

-This experiment explored the possible role of an artifact of the surgical procedure in the development of scoliosis in pinealectomized chickens.

-No significant differences in the incidence or severity of scoliosis were detected between four different pinealectomy techniques.

-Scoliosis developed after the pineal stalk was cut but not in the sham, cut skull, or control groups.

-Deliberate damage to the cerebrum adjacent to the pineal gland during the pinealectomy procedure or the presence of adhesions between the dura mater and cerebral cortex at the completion of the experiment did not produce scoliosis.

Chapter 4: The Effect of Pineal Transplantation on the Development of Scoliosis in Pinealectomized Chickens.

-This experiment explored the role of melatonin in the development of scoliosis in pinealectomized chickens.

-Pineal transplant is ineffective in altering the serum melatonin levels or the development of scoliosis in pinealectomized chickens.

-Serum melatonin levels did not differ between the group of pinealectomized chickens that developed scoliosis and the group that did not.

Chapter 5: The Development of Scoliosis Following Pinealectomy in two Breeds of Chicken with Different Rates of Growth.

-This experiment was designed to gain insight into the pinealectomy model and compare it to AIS.

-A higher growth velocity in Mountain Hubbard chickens is associated with a higher incidence and severity of scoliosis during early development.

-Growth rate may be an important factor in the development of scoliosis in pinealectomized chickens.

Chapter 6: The Influence of Gender on the Development of Scoliosis in Pinealectomized Chickens.

- This experiment was designed to gain insight into the pinealectomy model and compare it to AIS

-Females tend to develop more double curves than male chickens, but all other aspects of curve development are similar between the genders.

Chapter 7: The Immediate Effect of Light on Serum Melatonin Levels During the Dark Cycle in Young Chickens.

-This experiment was performed to verify the effect of red and white light on the peak serum melatonin levels in chickens.

-Exposure to red light for 60 minutes does not cause a significant decrease in serum melatonin levels in chickens, but exposure to white light causes a significant decrease within 20 minutes.

-Red light must be used during the blood collection procedure to ensure that the ambient lighting conditions to not alter the serum melatonin levels.

8.5 REVIEW OF THESIS HYPOTHESES AND OBJECTIVES

1. Determine if scoliosis is a result of the removal of the pineal gland or another aspect of the surgical procedure.

The results of this thesis indicate that scoliosis develops from the removal of the pineal gland rather than another aspect of the surgical procedure. Using four different techniques to remove the gland did not alter the development of scoliosis in these chickens and therefore a subtle aspect of the procedure does not appear to be the cause. Furthermore, a precise step during the surgery has been identified (cutting the pineal gland) which is necessary for the scoliosis to develop. Scoliosis does not develop until the pineal stalk is cut and does not become more severe or more common with further surgery. These findings provide evidence that the development of scoliosis is linked to the cutting of the pineal stalk rather than damage to the brain that may occur during the procedure. This is further substantiated by the fact that chickens with intentionally damaged brains in the region of the pineal gland do not develop scoliosis.

Chickens that underwent the pinealectomy procedure commonly developed adhesions between the dura mater and cerebral cortex. However, there was no relationship between the incidence of these adhesions and scoliosis.

While these experiments do not provide definitive proof that the scoliosis does not result from an aspect of the surgery other than the removal of the pineal gland, they do add to the collection of evidence that supports that belief.

2. Does scoliosis in pinealectomized chickens result from low levels of serum melatonin?

The results of this thesis indicate that serum melatonin levels by themselves are poor indicators of scoliosis development in pinealectomized chickens. All pinealectomized chickens had low serum melatonin levels and there were no significant differences in serum melatonin levels between those chickens that developed scoliosis and those that did not. If low serum melatonin were the critical factor in the development of scoliosis then all chickens with low serum melatonin levels would be expected to develop scoliosis but only about 60% of those chickens developed scoliosis. It is possible that another factor, alone or in conjunction with low serum melatonin levels, is responsible for the development of scoliosis in pinealectomized chickens.

Like the two phases that have been proposed in the development of AIS, there may be two phases in the development of scoliosis in pinealectomized chickens: Phase 1 - Initiation of the curve (Independent of serum melatonin levels). Phase 2 - Progression of the curve (Associated with low serum melatonin levels). It is possible that the initiation of the curve is genetically determined, with approximately 60% of chickens being susceptible. When serum melatonin levels are reduced following pinealectomy it may be those 60% that develop curves. This hypothesis is supported by human studies. AIS is known to exhibit a familial pattern indicative of a genetic linkage (Wynne-Davies 1968). Furthermore, low levels of serum melatonin may be involved in the development of scoliosis because girls with progressive scoliosis have been found to have lower serum melatonin levels than non-progressive patients or controls (Machida *et al.* 1996). It is possible that the same etiology and pathogenesis are involved in both the development of scoliosis in pinealectomized chickens and AIS.

3. Provide Insight into the Relevance of the Model to AIS

It has been established that scoliosis develops following pinealectomy which has many similarities to curves seen in AIS (Machida *et al.* 1994;

Kanemura *et al.* 1997; Wang *et al.* 1997). The experiments of this thesis identified two additional similarities between the two phenomena. First, as in AIS there is a significant effect of gender on the development of scoliosis in pinealectomized chickens. Although the effect is not as pronounced as seen in AIS, female pinealectomized chickens develop a significantly higher incidence of double curves compared to male pinealectomized chickens. Second, the development of scoliosis in linked to growth in pinealectomized chickens as it is in AIS. This finding is exciting because GH has been proposed to be involved in the etiology of AIS (Ahl *et al.* 1988) and these findings suggest that it may also be involved in the development of scoliosis in pinealectomized chickens.

8.6 DIRECTIONS FOR FUTURE RESEARCH

There is an increasingly large body of evidence that implicates growth and growth hormone in the development of scoliosis in pinealectomized chickens and in AIS. Two experiments to examine the role of GH are proposed.

1. Determining serum GH in addition to serum melatonin in pinealectomized chickens would help clarify the role of GH in the phenomenon. The results of this thesis did not detect any difference in the percentage growth rates between chickens that developed scoliosis and those that did not during

weeks 2 or 3, but the difference in GH may occur prior to week 2 or may not translate into significant differences in size.

2. To understand the role of GH and melatonin in AIS we propose to measure the levels of these hormones during the initial stages of curve development when they are proposed to differ compared to controls.

3. Several authors have been able to significantly reduce serum melatonin levels by non-surgical means. These tehhniques have included the use of bright light (McIntyre *et al.* 1989), β-blockers such as propranolol or atenolol (Nathan *et al.* 1997) or tryptophan deficient diets (Zimmerman *et al.* 1993).

   Attempts at reducing serum melatonin with light for prolonged periods in chickens have proven ineffective (Bagnall *et al.* Unpublished). β-blockers are not likely to be effective at reducing serum melatonin in chickens because ß -receptors do not activate melatonin production in avian species (Blinkley *et al.* 1975; Blinkley *et al.* 1978).

   Tryptophan is the essential amino acid precursor of melatonin and a tryptophan deficient diet is the preferred technique to reduce serum melatonin in chickens by non-surgical means.

   Reducing serum melatonin in chickens by non-surgical means would determine if the scoliosis resulted from low levels of serum melatonin and may give insight into the role of the surgical procedure. For example if scoliosis does develop it can be definitively concluded that the scoliosis is not an artifact of the surgical removal of the pineal gland.

4. Differences in hormonal control and interactions are vast between mammalian and avian species which complicates the comparison of GH and melatonin between chickens and humans. Ideally a mammalian animal model would be preferred but pinealectomy has been performed in quadrupedal mammals without the development of scoliosis (O'Kelly *et al.* 1999). The lack of scoliois production was likely due to biomechanical and structural differences between quadruped and biped spines. It is believed that bipedal (or at least pseudo-bipedal) animals are required for the curve to develop. Therefore it is proposed to explore the results of pinealectomy in monkeys.

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Appendix A

Pinealectomy Procedure

At three to five days of age, each chicken was anesthetized using halothane and the pineal gland was removed together with its long stalk. This was achieved by making a small incision through the skin directly above his superior sagittal sinus and extending the incision posteriorly to just below the confluence of sinuses. The flaps of skin were separated to expose the skull. The pineal gland of the chicken lies directly beneath the confluence of sinuses on the surface of the brain. Using a pair of dissecting scissors, an incision was made through the skull in the shape of an inverted U. The tails of the incision were extended across the transverse sinuses so that a flap of soft bone could be pulled back to expose the pineal gland which was often firmly attached to the dura mater. Care was taken not to damage the gland as the flap of bone was pulled back. The skull bones are sufficiently flexible at this stage that the can be pulled back of a single flap and replaced easily after surgery. Using a pair of forceps and scissors, the pineal gland and its stalk were removed as a single unit and placed on a piece of gauze, where they were closely examined using a dissecting microscope to determine that the pineal gland was intact and that all of it had been removed, including the stalk.

The flap of skull was replaced gently and the skin sutured with four-zero suture. The chicken was removed from the anesthetic and kept under a warm lamp for 24 hours to ensure complete recovery, which usually occurred with one within one hour as evidenced by the chicken starting to eat and drink again. The chickens were then returned to their normal housing conditions. This technique is similar to that described by both Thillard (1959) and Machida *et al.* (1993, 1994, 1995, 1997)

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Appendix B

Protocol for Melatonin Radioimmunoassay

Solutions required (Use fresh, glass-distilled water for all reagents)

Tricine Buffer

17.9 g Tricine (Sigma)
9.0 g NaCl (ASC)
1.0 g Sodium azide (NaN_3) (Sigma)
1.0 g Gelatine (ASC)
Water to 1 litre

Stir solution continuously and heat to 50°C (or less) for 15 min. to dissolve gelatine

Cool to room temperature.

Adjust pH to 8.0 (from ~ 5.5) with 4 N NaOH.

N.B. Do not heat to > 50°C !

Can be stored at 4°C for up to one week.

Antiserum:

Supplied freeze-dried from Stockgrand Ltd.
Reconstitute with 2 ml double-distilled water (1:10 dilution).
Aliquot into 22 ml portions and store at – 20°C
Working solution : (for 200 tubes)
Dilute a 22 µl aliquot into 20 ml tricine buffer (1:9000 dilution)

Label:

[^{3}H] Melatonin (NEN) (specific activity ~ 80 Ci/mmol, conc. 1 mCi/ml)
Dilute this 1:100 with ethanol and store at – 20°C
Working solution:
Dilute with 22 ml tricine buffer (for up to 220 tubes).

Standards (stock):

1 mg Melatonin (Sigma) in 1 ml ethanol (1 mg/ml) **(A)**
Dilute 50 (ml) l (A) to 950 ml tricine buffer (50 µg/ml) **(B)**
Dilute 40 ml (B) to 960 ml tricine buffer (200 ng/ml) **(C)**
Dilute 1 ml (C) to 99 ml tricine buffer (2.0 ng /ml) **(D)**
Prepare 125 ml aliquots of (D) and store at – 20° (Stock)

Standards (working): (In 13x 100 mm tubes)

Thaw 1 vial of melatonin stock to do 1 duplicate set of standard curves
Add 100 µl stock to 1900 µl tricine buffer. Vortex. (Tube 1)
Prepare 9 additional tubes, each with 1000 µl tricine buffer (Tube 2)
Transfer 1000 µl from tube 1 to tube 2. Vortex.
Repeat (Standard serial dilution)

Pipette 250 µl from each tube (in duplicate) into 12 x 75 mm tubes for assay.
Melatonin concentrations:

Tube pair	#1	#2	#3	#4	#5	#6	#7	#8	#9	#10
pg/ml	1000	500	250	125	62.5	31.5	15.6	7.8	3.9	1.95
pg/tube	250	125	62.5	31.5	15.6	7.8	3.9	1.95	0.97	0.48

Samples

Spin coagulated blood from animals in centrifuge for 20 min. at 3000 rpm at 4°C

Pipette out all the serum and store at –20°C

Thaw the sample on ice when used

Dextran-coated charcoal: (Prepared fresh daily)

Acitivated charcoal (Sigma #C5260), 100-400 mesh

Dextran D-1390 (Sigma) (avg. molecular weight 72,600)

Suspend charcoal at 0.5% in tricine buffer (0.5g in 100 ml buffer)

Add 0.05% dextran (0.05g dextran)

Stir 30 min. at 4°C

Use immediately

MELATONIN RIA

1. prepare in duplicate:

	Buffer	Sample	Antibody	[^{3}H] Melatonin	Final Volume
Total Radioactivity	850 µl	–	–	100 µl	950 µl
NSB	350 µl	–	–	100 µl	450 µl
Total Binding	250 µl	–	100 µl	100 µl	450 µl
Standards	–	250 µl	100	100 µl	450 µl
Samples	150 µl	100 µl	100 µl	100 µl	450 µl

2. Vortex. Cover with foil and set in fridge to incubate overnight.

Next morning...

3. Make up dextran-coated charcoal
4. Begin timer. Add 500 µl charcoal to all tubes **except Totals.** (Making final volume 950 µl for all tubes)
5. Vortex 1 sec. Incubate 15 min. at 4°C
6. Centrifuge 15 min., 4°C, 3000 rpm **except Totals**

7. Remove 750 µl supernatant and 750 µl from Totals tubes to scintillation vials
8. Add 7.5 ml scintillation fluid, shake, count.

Appendix C

Analysis of the Reliability and Validity of the Serum Melatonin Assay

C.1 INTRODUCTION

A competitive binding ^{3}H-melatonin assay has been used to measure the serum melatonin levels of each chicken. The differing serum melatonin results in humans (Bagnall *et al.* 1996; Machida *et al.* 1996) and chickens (Machida *et al.* 1995; Wang *et al.* 1998) has led to questions of the reliability and validity of the technique. As a result, this study was undertaken to determine the reliability and validity of the technique and try to isolate the sources of error within the procedure.

C.2 METHODS

Fifty, newly-hatched chickens were obtained from a local hatchery (Lilydale, Edmonton). They were immediately introduced into a 12:12 light / dark cycle (dark 9:00am – 9:00pm) and provided food and water *ad libitum*. They were housed in a single pen under constant environmental conditions (26°C, 70% relative humiduty). During the middle of the light cycle on the 5th day after hatching, blood was collected from twenty-five chickens selected at random. Blood was collected from the remaining twenty-five chickens during the middle of the dark cycle on the 6th day after hatching.

Blood Collection

The blood collection procedure consisted of injecting the chicken with an overdose of euthanyl, followed immediately by opening the chest cavity and withdrawing 1.5 ml of blood from the heart using a #18-gauge needle. The blood was then immediately transferred to a non-heparinized glass tube and left at room temperature for four hours to allow coagulation to occur and then stored at 4°C for 24 hours. The blood samples were cryo-centrifuged for 20 minutes at 3000 rpm at 4° C and the serum was then pooled, vortexed, and then stored at -20° C until assayed within two weeks.

Serum Melatonin Assay

Serum melatonin levels were assessed using a competitive binding ^{3}H-melatonin assay (Appendix B). Four assays were undertaken in total, assays 1-3 were replicate assays to test the amount of variability present when the same sample was assayed repeatedly.

Assay #1,2,3

Blood collected in the middle of the dark cycle	30 replicates
Blood collected in the middle of the light cycle	30 replicates

These three assays indicated the intra-assay variation present while measuring identical samples, and showed the level of inter-assay variability between separate assays.

Assay #4

500 pg/ml standard melatonin solution	30 replicates
250 pg/ml standard melatonin solution	30 replicates

This assay determined amount of intra-assay variation present when standard melatonin solutions are used. Comparison of the variation present in the standard melatonin versus the serum sample indicated the error that can be attributed to the serum. This assay also showed the accuracy of the assay to measure a known amount of melatonin.

Testing the Accuracy of the Liquid Scintillation Counter (LSC)

To test the error of the LSC (LBK Wallac 1209 Rackbeta), 10 sample tubes were selected at random and sent through the LSC a total of 10 times. A similar test was undertaken with 10 tubes that contained a standard melatonin solution rather than serum. The mean and standard deviation for repeated measures of each tube were calculated to determine the variation in the assay procedure that can be attribute to the LSC. Differences in the error (SD as % of mean) between tubes containing serum and those containing standard melatonin were tested using ANOVA followed by Fisher's PLSD ad hoc test with the level of significance set at $p<0.05$.

C.3 RESULTS

Comparison of the average serum melatonin levels between individual assays as seen in Figures C-1 and C-2. For the sample collected in the dark the average values for assay #2 and #3 differed significantly from the average serum melatonin level of assay #1 (Figure C-1). The average serum melatonin levels of the sample collected in the dark did not differ significantly between the three separate assays (Figure C-2).

The amount of variation in measuring the serum melatonin level of sample repeatedly is significant. The standard deviation is approximately 50% of the mean in all cases (Table C-1, Table C-2). This trend is consistent between the samples collected in the dark, the samples collected in the light and the samples that contained standard melatonin.

The results of assay #4 are given in Table C-2. For the 30 replicates containing 250 pg/ml the average concentration determined by the assay was 340.2 ± 168.1 pg/ml. For the tubes containing 500 pg/ml the average concentration of melatonin determined by the assay was 444.6 ± 173.3 pg/ml. Despite the lack of accuracy and the large variation, the assay was able to detect a significant difference between these two groups.

Testing the Error Due to the Liquid Scintillation Counter

The mean, standard deviation, and standard deviation as a percentage

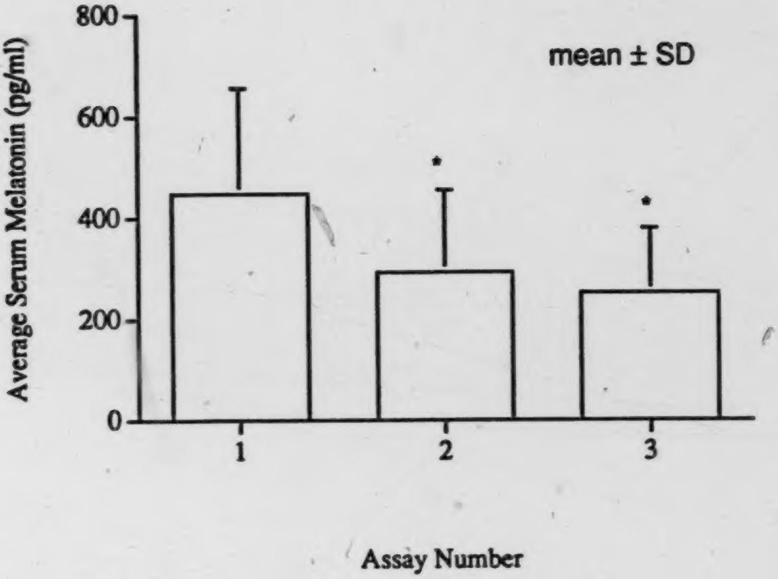

Figure C-1. The average serum melatonin levels for the serum sample collected in the dark for three separate assays. The average serum melatonin levels in assay #2 and assay #3 are significantly lower that the average serum melatonin levels of assay #1 (significant difference denoted by the asterisk (*)).

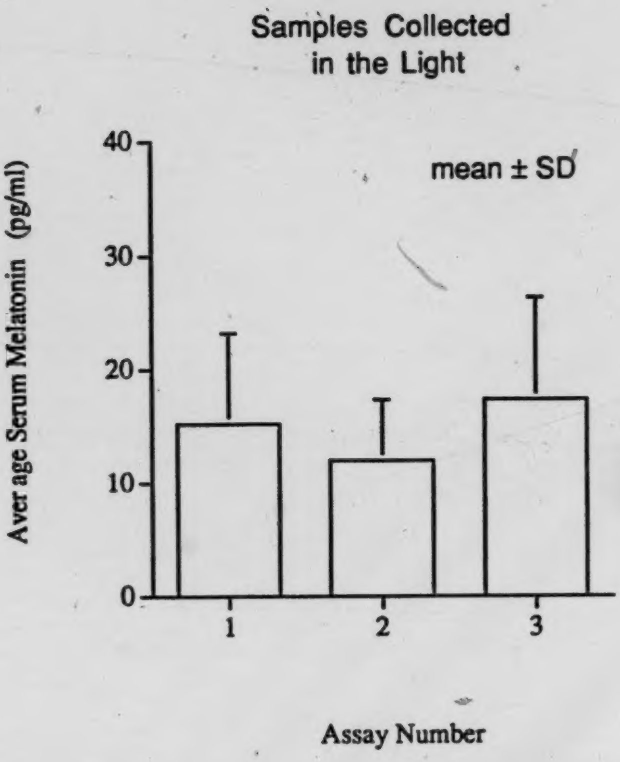

Figure C-2. The average serum melatonin levels for the serum sample collected in the light for three separate assays. The average serum melatonin levels did not differ significantly between the three assays.

Table C-1. The Variation Present when Repeatedly Measuring the Same Serum Sample for Melatonin Content

Group	Assay #	n	Mean (pg/ml)	SD (pg/ml)	SD as a Percent of Mean
Dark	1	30	448	208	46%
	2	30	293	162	55%
	3	30	251	128	51%
Light	1	30	15	8	53%
	2	30	12	5	42%
	3	30	17	9	53%
average					50%

Table C-2. The Accuracy of the Melatonin Assay Using Standard Melatonin

Group	n	Mean (pg/ml)	Percent Difference to Actual Value	SD (pg/ml)	SD as Percent of the Mean
250 pg/ml	30	340.2	-11%	168.1	49%
500 pg/ml	30	444.6	36%	173.3	39%
p-value		0.02			

of the mean are presented in Table C-3 for the ten tubes containing serum that were measured 10 times by the LSC. The standard deviation as a percentage of the mean ranged from 10%-22% and averaged 18%. Therefore of the approximately 50% error of the technique, almost 2/5 (18% / 50%) of that error is due to the variation in the machine reading the tubes.

The LSC produced less error when repeatedly measuring tubes containing only standard melatonin (Table C-4). The standard deviation as a percentage of the mean ranged from 0-13% with an average of 7%. This was significantly less error compared to the 18% in the repeated measures of tubes containing serum.

C.4 DISCUSSION

The results of this study show that the serum melatonin assay has a limited ability to determine differences between samples. The large standard deviation present when assaying an identical sample numerous times indicates that the technique has a low level of precision. The serum melatonin level must be considered to fall within the range determined by the error rather than being a precise value. Therefore the technique does not allow for the differentiation of small differences in serum melatonin levels and is best suited to determine differences between group values rather than individual samples. However, differences in serum melatonin levels between groups that are larger than the error associated with the technique can be determined. Given this fact, when the assay is able to detect significant

Table C-3. Testing the Liquid Scintillation Counter by Repeated Measurement of Tubes Containing Serum Melatonin

Tube	n	Mean	SD	SD as a percentage of Mean
a	10	50	6.5	13%
b	10	13	2.5	19%
c	10	24	4.6	19%
d	10	11	2.1	19%
e	10	7.7	1.5	19%
f	10	8	1.7	21%
g	10	21	4.7	22%
h	10	8.4	0.8	10%
i	10	11	1.7	15%
j	10	12	2.2	18%
Average				18%

Table C-4. Testing the Liquid Scintillation Counter by Repeated Measurement of Tubes Containing Standard Melatonin

Tube	n	Mean (pg/ml)	SD (pg/ml)	SD as a percentage of Mean
a	10	0	0	0%
b	10	0	0	0%
c	10	791	84	11%
d	10	1008	129	13%
e	10	107	11	10%
f	10	6	0.4	6%
g	10	383	29	8%
h	10	636	49	8%
i	10	485	40	8%
j	10	528	34	6%
average				7%

differences between the average serum melatonin levels of groups the difference between those groups must have been quite substantial.

The result of assay #4 shows the accuracy of the technique. The tubes that were known to contain 500 pg/ml were measured to be 11% below that value on average. On the other hand, the tubes that were known to contain 250 pg/ml were measured to be 36% higher than the actual value. It therefore appears that the accuracy of the assay to determine the exact level of melatonin in a sample is limited.

Considering the large degree of variability between the means in assays #1 - #3, the values attained in a single assay can not be compared directly to serum melatonin levels from other assays. The standard deviation of samples is similar between assays, and if the distributions were assumed to be normal the serum melatonin levels could be converted to a Z-score for comparison between assays. However, the distribution of samples can not be assumed normal in all cases. Therefore the comparison of serum melatonin levels between assays may not be valid.

Sources of Error

Approximately 2/5 of the 50% variation around the mean of serum samples can be attributed to the LSC. Small differences in "counts" are translated into relatively large differences in serum melatonin levels by the standard curve equation, which magnifies the error of the counting device. The remaining 3/5 $^{\text{ths}}$ of the error can be attributed to limitations of the measuring devices (pipettes etc) and humans error. Other authors have reported much less error when using radioimmunoassay procedures (Machida *et al.* 1996) or HPLC techniques (Hilibrand *et al.* 1996) to measure melatonin. The use of these alternative techniques is currently being explored.

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